The role of host cell factors in the lytic reactivation of Kaposi's sarcoma-associated herpesvirus from latency

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Declaration

I, Lucy Dalton-Griffin confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated.

Signed:.....
Date:.....

Abstract

Kaposi's Sarcoma-Associated Herpesvirus (KSHV) has two stages to its life cycle; latency and lytic replication. KSHV latent infection is associated with the B-cell tumour, Primary Effusion Lymphoma (PEL). During latency the viral episome is maintained, few viral genes are expressed and no infectious virions are produced. The switch between phases is controlled by the viral transcription factor, RTA encoded by ORF50. Lytic replication, which results in the production of progeny virions, can be triggered by a variety of causes suggesting KSHV has the ability to reactivate in response to a multitude of scenarios. However, many of the stimuli described, to date, have no clear physiological relevance.

This thesis investigates how cellular transcription factors can induce the KSHV lytic cycle. Firstly, a model system for monitoring KSHV lytic replication is developed and characterised. This system is then employed to demonstrate how the cellular transcription factor XBP-1s is able to induce KSHV reactivation. XBP-1 is responsible for the terminal differentiation of B-cells into plasma cells (PCs) and is a major regulator of the unfolded protein response (UPR). PEL do not express the transcription factor X-box binding protein-1 (XBP-1). When spliced active XBP-1s is supplied, PEL cells differentiate towards a PC and induce KSHV lytic replication. B-cell terminal differentiation is therefore a physiological trigger of KSHV lytic reactivation. To understand the role of host cell factors in this interaction further, the gene expression changes that occur when XBP-1s is supplied to PEL are also examined. Finally, we investigate the specific roles of XBP-1s and HIF-1α in the KSHV reactivation seen in response to hypoxia. We conclude that both B-cell differentiation and hypoxia are physiologically relevant triggers for KSHV lytic cycle induction, highlighting some of the many complex interactions between the virus and its host.

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Abbreviations

(Ψ)	Packaging sequence
Ag	Antigen
AGM	African green monkey
Ago	Argonaute
AID	Activation induced deaminase
AIDS	Acquired immunodeficiency syndrome
AIDS-KS	AIDS-associated KS
aHIF	Antisense HIF
AP-1	Activating protein 1
APC	Antigen presenting cell
APR	Acute phase response
ARCN-1	Archain 1
ARF	Alternate reading frame
Arnt	Aryl-hydrocarbon receptor nuclear translocator
ASK1	Apoptosis signal-regulating kinase 1
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BCBL	Body cavity-based lymphoma
BCL	B-cell lymphoma
BCLAF-1	BCL-2-associated transcription factor 1
BCR	B-cell receptor
BECs	Blood vessel endothelial cells
bHLH	Basic helix-loop-helix
BiP	IgH binding protein
BL	Burkitt's lymphoma
BLIMP-1	B-lymphocyte-induced maturation protein-1
bp	Base-pair
BSAP	B-cell specific activator protein
bZIP	Basic leucine zipper
CA9	Carbonic anhydrase 9
C/EBPα	CCAAT/enhancer binding protein alpha
CBP	CRE-binding protein
CCND1	Cyclin D 1
CCNE	Cyclin E
CD	Crohn's Disease
cDNA	Complementary DNA
cdk	Cyclin dependent kinase
cdki	cdk inhibitor
chIP	Chromatin immune-precipitation
CHOP	CAAT/enhancer binding protein, homologous protein

CIRBP	Cold inducible RNA binding protein
CL5/9	rKSHV.219 infected HEK293T clonal cell line 5/9
CO_2	Carbon Dioxide
COPE	Coatomer protein complex subunit epsilon
CRE	Cyclic AMP response element
CREB-H	CRE binding protein - hepatocyte
Cryo-EM	Cryogenic electron microscopy
CSGW	cPPT-SFFV-EmGFP-WPRE
CSPW	cPPT-SFFV-PAC-WPRE
CSR	Class switch recombination
C-TAD	C-terminal transcriptional activation domain
CTD	C-terminal domain
CTL	Cytotoxic T-lymphocyte
CXCR	CXC motif chemokine receptor
CXCL	CXC motif chemokine ligand
D	Ig diverse region
DC	Dendritic cell
DE	Delayed early
DISC	Death inducing signalling complex
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DMVEC	Dermal microvascular endothelial cell
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleotide triphosphate mix
DDOST	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
DTT	Dithiothreitol
DUSP	Dual specific phosphatase
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDEM	ER degradation enhancer mannosidase-α like 1
EDTA	Ethylenediaminetetraacetic acid
eIF-2α	Eukaryotic initiation factor- 2α
EIF2C1	Eukaryotic translation initiation factor 2C 1
EmGFP	Emerald GFP
EPAS	Endothelial PAS domain protein
EPO	erythropoietin
ER	Endoplasmic reticulum

ERAD	ER-associated protein degradation
ERK	Extracellular signal regulated kinase
ERO	ER oxidoreductins
ERSE	ER stress response element
FACS	Fluorescence activated cell sorting
FADD	Fas associated death domain
FAK	Focal adhesion kinase
Fc	Fragment crystallisable
FCLR4	Fc receptor like 4
FCS	Foetal calf serum
FDCs	Follicular dendritic cell
FIH	Factor inhibiting HIF
FKBP2	FK506 binding protein 2
FL	Follicular lymphoma
FLICE	FADD interleukin-1β-converting enzyme
FLIP	FLICE inhibitory protein
FOXP1	Forkhead box P1
FRP-1	Fusion regulation protein 1
fwd	Forward
GADD	Growth arrest DNA damage gene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Germinal centre
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GLRX	Glutaredoxin
GLS	Golgi localisation signal
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte macrophage colony stimulating factor
GOLGB1	Golgi autoantigen B1
GOLT1B	Golgi transport 1B
GORASP2	Golgi reassembly stacking protein 2
GOSR2	Golgi SNAP receptor complex member 2
GPCR	G-protein coupled receptor
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine 5’ triphosphate
HAART	Highly active antiretroviral therapy
HAS	HIF-1α ancillary sequence
HAT	Histone acetyl transferase
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HDAC	Histone deacetylase

HEK	Human embryonic kidney
HERPUD1	Homocysteine-inducible ER stress-inducible Ub-like domain member 1
Hey1	Hairy/E(spl) related with YRPW
HHV	Human-herpesvirus
HIF	Hypoxia inducible factor
HIS	Histidine
HIV-1	Human immunodeficiency virus-1
hKFC	Ste20 like kinase
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
HNF4	Hepatocyte nuclear factor 4
HPh	Hygromycin phosphotransferase
HPV	Human papillomavirus
HRE	Hypoxia response element
HRP	Horse radish peroxidase
Hsp	Heat shock protein
HSV	Herpes-simplex virus
hTERT	Human telomerase reverse transcriptase
HUVEC	Human umbilical vein endothelial cell
HVA	Herpesvirus ateles
HVS	Herpesvirus saimiri
HYOU1	Hypoxia upregulated 1
IBD	Inflammatory bowel disease
ICN	Intracellular domain of NOTCH
IE	Immediate early
Ig	Immunoglobulin
IgH	Ig heavy chain
IgL	Ig light chain
IKK	Inhibitor of kappa β kinase
IL	Interleukin
IPAS	Inhibitory PAS
IRE-1α	High inositol-requiring kinase/endonuclease protein 1α
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
IRS	Insulin receptor substrate
IS	Immunological synapse
ISR	Integrated stress response
ISRE	Interferon-stimulated response element
ITAM	Immunoreceptor tyrosine based activation motif
IU	Infectious units
J	Joining

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kb	Kilo base
K	KSHV unique gene
KCP	KSHV complement control protein
kDa	Kilodaltons
KDEL-R2	Lys-Asp-Glu-Leu ER retention receptor
K-RBP	KSHV RTA binding protein
KS	Kaposi's sarcoma
KSHV	Kaposi’s sarcoma-associated herpesvirus
L	Late
LANA	Latent nuclear antigen
LANA-2	Latency-associated nuclear antigen-2
LAT	Latency-associated transcript
LB	Luria-Bertani
LECs	Lymphatic endothelial cells
LEF	Lymphoid enhancing factor
LMAN1	Lectin mannose binding protein 1
LMP	Latent membrane protein
Log(2)	Log base 2
LPS	Lipopolysaccharide
LR	Leucine heptapeptide repeat
LTR	Long terminal repeat
LUR	Long unique region
MAN1A2	Manosidase-1A2
MAPK	Mitogen-activated protein kinase
MCD	Multicentric Castleman’s disease
MCP	Major capsid protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibiity complex
MHV-68	Murine gammaherpesvirus 68
MIR	Modulators of immune recognition
mRNA	Messenger RNA
miRNA	Micro RNA
MITF	Micropthalmia-associated transcription factor
MK2	MAP K 2
MM	Multiple myeloma
MOI	Multiplicity of infection
MTA	mRNA transcript accumulation protein
MTA3	Metastasis-associated 1 family member 3
mTOR	Mammalian target of Rapamycin
MuHV-4	Murine herpesvirus 4

MVECs	Microvascular endothelial cells
MZ	Marginal zone
N_2	Nitrogen
NaBut	Sodium butyrate
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NHL	Non-Hodgkin lymphoma
NLS	Nuclear localisation signal
nm	nanometre
NO	Nitric oxide
NPC	Nuclear pore complex
NRF2	Nuclear factor erythroid 2 (NF-E2)-related factor 2
N-TAD	N-terminal transcriptional activation domain
O_2	Oxygen
OAS	2'-5' oligoadenylate synthase
Oct	Octamer binding protein
ODD	Oxygen dependent degradation domain
ORF	Open reading frame
Ori-Lyt	Origin of lytic replication
P/S	Penicillin and streptomycin
PAc	Puromycin glycol
PALS	Periarteriolar lymphoid sheath
PAN	Polyadenylated nuclear transcript
PARP	Poly (ADP-Ribose) polymerase
PAS	Per-Arnt-Sim domain
PAX5	Paired box protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
PEG	Polyethylene glycol
PEL	Primary effusion lymphoma
PERK	PKR-like ER kinase
PF-8	Processivity factor 8
PHD	Prolyl hydroxylase
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKR	Double-stranded DNA dependent protein kinase
PLDN	Pallidin homolog
PP1	Protein phosphatase 1

PPIB	Peptidyl isomerise B
Prdm1	Positive-regulatory-domain-containing 1
pre-B-cells	Precursor B-cell
Pre-BCR	Pre-B-cell receptor complex
Pre-mRNA	Precursor mRNA
Pre-miRNA	Precursor miRNA
Pro-B-cells	Progenitor B-cell
PTB	Polypyrimidine tract binding protein
PTEN	Phosphatase and tensin homolog
PTP-1B	Protein tyrosine phosphatise 1B
RACK	Receptor of activated protein kinase C
RAG	Recombinase-activating gene
RAP	Receptor associated protein
Rb	Retinoblastoma
Rbl2	Rb-like protein 2
RBP-Jκ	Recombination-signal binding protein Ig Jκ region
RDA	Representational difference analysis
redox	Reduction-oxidation
rev	Reverse
RFHV	Retroperitoneal fibromatosis-associated herpesvirus
RGD	Arginine glycine aspartate domain
RIG-I	Retinoic acid inducible gene 1
RIN	RNA integrity number
RIP	Receptor Interacting protein
RLU	Relative light unit
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RPN1	Ribophorin 1
RRE	RTA response element
RRV	Rhesus Rhadinovirus
RT	Reverse transcriptase
RTA	Regulator of transcription activation
S1P	Site 1 protease
S2P	Site 2 protease
SDF-1	Stromal cell-derived factor-1
SDR	Thioltransferase dehydrogenase/reductase
SDS	Sodium dodecyl sulphate
SFFV	Spleen focus-forming virus

SHM	Somatic Hypermutation
shRNA	Short hairpin RNA
SIN	Self-inactivating
siRNA	Small/short interfering RNAs
SLC	Surrogate light chain
SNP	Single nucleotide polymorphism
SOX	Shutoff and exonuclease
SP-1	Specificity protein 1
SPC	Signal peptidase complex
SRP	Signal recognition protein
ssDNA	Salmon sperm DNA
SSR	Signal sequence receptor
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
TAE	Tris-acetate-EDTA
Tat	HIV tat
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBST	TBS tween
TCR	T-cell receptor
TdT	Terminal deoxynucleotide transferase
TE	Tris-EDTA
Th	T- helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Topo	Topoisomerase
TPA	12-0-tetradecoylphorbol 13-acetate
TR	Terminal repeats
TRAF	Tumour necrosis factor receptor-associated factor
TRAM1	Translocation associated membrane protein 1
TRIM	Tripartite motif containing protein
tRNA	Transfer RNA
Ub	Ubiquitin
UC	Ulcerative Colitis
UCP	Ub carrier protein
UDG	Uracil DNA glycosidase
UK	United Kingdom
UPR	Unfolded protein response
UPRE	UPR element
USA	United States of America
UTR	Untranslated region
UV	Ultra-violet

V	Ig variable region
V1/2	Variable region 1/2
vBcl-2	Viral Bcl-2
v-Cyc	Viral cyclin
VDP	Vesicle docking protein
VEGF	Vascular endothelial growth factor
vFLIP	Viral FLICE inhibitory protein
vGPCR	Viral G-protein coupled receptor
VHL	Von Hippel Lindau
vIL-6	Viral IL-6
vIRF	Viral interferon regulatory factor
vMIP	Viral macrophage inflammatory protein
vOX-2	Viral OX-2
VZV	Varicella-Zoster virus
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
XBP-1	X-box binding protein-1
XBP-1s	Spliced XBP-1
XBP-1u	Unspliced XBP-1
xCT	Cysteine/glutamate transporter
XRE	XBP-1 response element

1 Introduction

Viruses are ultramicroscopic, obligate intracellular parasites and therefore, by definition, are reliant on and intricately associated with the host cell for their replication. Through co-evolution, most aspects of virus biology are shaped by its interaction with the host, to allow continued success of these host dependent microorganisms. However, in the past, a new virus discovery was often followed by intense focus on disease association and the virus itself, often in isolation of the host, with emphasis given to the virus structure and viral genes encoded. Later, more in depth study revealed many important host-pathogen interactions including the interferon response and the use of cellular receptors for virus entry. More recently, the development and application of systems biology and genomic approaches have allowed these interactions to be investigated further (reviewed in (Peng et al., 2009) (Bailer and Haas, 2009)). These integrative methods can provide a multidimensional view of virus-host interactions. Problems to overcome include the development of models to make sufficiently accurate interaction predictions and the requirement of interdisciplinary collaborations. Despite this, these high-throughput techniques highlight the importance of studying viruses in the context of host infection. Studies have already demonstrated that different viruses adopt similar core interactions in order to by-pass or hi-jack the cellular machinery and responses (Fossum et al., 2009). We have also seen how these interactions can inadvertently cause disease, for example the human herpesvirus Kaposi's sarcoma-associated virus (see section 1.1.8) (Moore and Chang, 2003) (Dalton-Griffin and Kellam, 2009). Consequently, understanding virus-host interactions and their complexities has far reaching implications for translational research and drug discovery.

1.1 Kaposi's Sarcoma-Associated Herpesvirus (KSHV)

Kaposi's Sarcoma-associated Herpesvirus (KSHV) (human herpesvirus 8; HHV8) is the aetiological agent of Kaposi's Sarcoma (KS) (Chang et al., 1994), a malignancy that accounts for 10 % of all cancers in areas of sub-Saharan Africa and is the most common cause of cancer in African men (Schwartz et al., 2008) (Hengge et al., 2002) (Parkin et al., 1999). Additionally, the B-cell lymphoproliferative disease primary effusion lymphoma (PEL) is also associated with KSHV infection (Cesarman et al., 1995), and represents 4 % of all acquired

immunodeficiency disease (AID) related non-Hodgkin lymphomas (Carbone and Gloghini, 2008). KSHV is thus the most common cause of cancer among those with untreated HIV infection (Beral et al., 1990) (Boshoff and Weiss, 2002) (Casper and Wald, 2007). These diseases are most likely caused, unintentionally, by the mechanisms employed by KSHV to evade the host immune system and establish latency. These interactions, although necessary for virus propagation, can lead to cell proliferation and tumorigenesis in a susceptible individual. The entire life cycle of KSHV is intertwined with its host including both latent and lytic stages as well as, importantly for the topic of this thesis, the switch between the two phases, reactivation (Hayward, 1999).

KSHV was discovered in 1994 by Chang *et. al*. using the technique of representational difference analysis (RDA) (Chang et al., 1994). This study indentified a potential infectious aetiology for the tumour of endothelial origin, Kaposi Sarcoma, originally described by Mauritz Kaposi in 1872 as an 'idiopathic multiple pigmented sarcoma of the skin' (Kaposi, 1872). KSHV or human herpesvirus 8 (HHV8) is the eighth and most recently identified human herpesvirus.

1.1.1 Herpesviruses

There are currently 90 species of herpesviruses (order - *Herpesvirales*) recognised (www.ICTVonline.org), all are large double stranded DNA viruses with genomes typically between (125-290kb) and with a characteristic virion structure (Davison et al., 2009). These viruses are highly adapted to their host, often through millions of years of co-evolution (Hayward, 1999) and can be divided into three distinct family groups depending on the host range (McGeoch et al., 2006). The first group, *Herpesviridae*, contains those viruses infecting mammals, birds and reptiles naturally, the second group, *Alloherpesviridae*, infect amphibians and fish. Finally, the third group, *Malacoherpesviridae*, contains a single virus that infects the invertebrate bivalve molluscs (Davison et al., 2005). Herpesviruses have been historically identified by biological criteria, including cell tropism and replication cycle. As technology progressed, information concerning virus morphology and component parts became available and facilitated further categorisation. These details are used to divide the *Herpesviridae* into three subfamilies *Alpha-*, *Beta-*, and

Gammaherpesvirinae (Roizman et al., 1981) (McGeoch et al., 2006). Classification is now carried out using the additional information provided by sequence-based phylogeny, and new genera have been added to the *Herpesviridae* family. This update of taxonomy includes the addition of two new genera to the *Gammaherpesvirinae* subfamily as well as new taxa to the *Alloherpesviridae* group (Davison et al., 2009) (reviewed in (Davison, 2010)).

The gamma-herpesviruses are characterised by a limited host range, lymphocyte tropism and sequence homology. KSHV is a gamma-2-herpesvirus (γ2-) and is the first member of the genus *Rhadinovirus* known to infect humans (Moore et al., 1996a) (Neipel et al., 1998). Rhadinoviruses have also been discovered in several animal species, including cattle, mice and primates (Rovnak et al., 1998) (Efstathiou et al., 1990). Phylogenetic analysis of the primate virus genomes has led to the identification of three separate Rhadinovirus lineages. Members of the first lineage are T-lymphotropic viruses infecting New World monkeys, including the archetypal Rhadinovirus, herpesvirus saimiri (HVS) and the closely related ateles herpesvirus (HVA). These viruses infect squirrel monkeys (*Saimiri sciureus)* and spider monkeys (*Ateles geoffryi)* respectively (reviewed in (Ensser and Fleckenstein, 2005)). Rhadinoviruses have also been identified in Old World primates such as chimpanzees (Greensill et al., 2000a), gorillas (Lacoste et al., 2000) and gibbons (Duprez et al., 2004). With the discovery of further primate Rhadinovirus genomes in African green monkeys (Greensill et al., 2000b) and Macaques (Desrosiers et al., 1997) it has been shown that there are two distinct lineages within the Rhadinoviruses that infect Old World monkeys. The first genogroup, RV1, is represented by KSHV and the retroperitoneal fibromatosis-associated herpesvirus (RFHV) that can cause a rare disease in Macaques, reminiscent of KS (Rose et al., 1997) (Bruce et al., 2006). The second group, RV2, contains the rhesus Rhadinovirus (RRV) and the novel gibbon virus HyloRHV2, no human virus has yet been found in this sub-group (Rose *et.al.* KSHV International Conference 2009). The most closely related human virus to KSHV is Epstein-Barr virus (EBV) from the *Lymphocryptovirus* genus.

1.1.2 Human Herpesviruses

Currently, there are eight identified human herpesviruses, all of which are associated with human disease (Table 1-1). Despite a considerable proportion of the population being infected with one or more herpesviruses, disease is usually only seen in the immunocompromised.

Table 1-1 Human Herpesviruses and their disease associations

Sub-family	***Human herpesvirus***	***Common name***	***Disease associations***
α	**HHV-1**	Herpes-simplex virus (HSV)-1	Oropharangeal herpes (cold sores)
			Genital herpes
α	**HHV-2**	Herpes-simplex virus (HSV)-2	Genital herpes
α	**HHV-3**	Varicella-Zoster virus (VZV)	Varicella (chickenpox)
			Zoster (shingles)
γ	**HHV-4**	Epstein-Barr virus (EBV)	Infectious mononucleosis
			Nasopharyngeal carcinoma
			Burkitt's lymphoma
			Classical Hodgkin's lymphoma
β	**HHV-5**	Human cytomegalovirus (HCMV)	CMV-mononucleosis
			CMV disease
β	**HHV-6a**		Exanthem subitum (sixth disease)
	HHV-6b		Roseola infantum
			Encephalitis
β	**HHV-7**		Exanthem subitum (sixth disease)
			Encephalitis
γ	**HHV-8**	Kaposi's sarcoma-associated herpesvirus (KSHV)	Kaposi's sarcoma
			Primary effusion lymphoma
			Multicentric Castleman's disease

1.1.3 KSHV genome

KSHV has a double stranded DNA (dsDNA) genome of 165 to 170 kilo bases (kb) (Renne et al., 1996a), consisting of a long unique region (LUR) approximately 140-145 kb in length which encodes all viral open-reading frames (vORFs) (Figure 1-1) (Russo et al., 1996). The LUR is flanked at each end by a series of non-coding GC-rich terminal repeats (TRs), which are 801 base pairs (bp) long. On average, isolates contain 20-25 (kb) of total TR DNA; however the number of repeats can range between 16-75; accounting for the variation seen in genome size between KSHV isolates (Lagunoff and Ganem, 1997). The genome exists as a circular dsDNA episome during latent KSHV infection

however, during lytic replication linear dsDNA genome is detected in reactivating cells and virions; by Gardella gel analysis (Renne et al., 1996a).

Comparison of the genome organisation and gene content of five gamma-herpesviruses, including KSHV, identified four major blocks of conserved genes (Nicholas et al., 1998). These genes are predominantly involved in replication or are structural components of the virus and many are also conserved in the alpha and beta-herpesviruses (Alba et al., 2001). There is also a high degree of similarity between the KSHV genome and the genomes of the primate viruses, retroperitoneal fibromatosis-associated herpesvirus (RFHV) and rhesus monkey Rhadinovirus (RRV). However, at the time of KSHV genome annotation the closest sequenced virus of the Rhadinovirus subfamily was herpesvirus saimiri (HVS). Therefore, many of KSHV's >90 ORFs were named using the logical HVS nomenclature. Any ORF with no homology to a HVS gene was assigned a K followed by a number in consecutive order relating to its location.

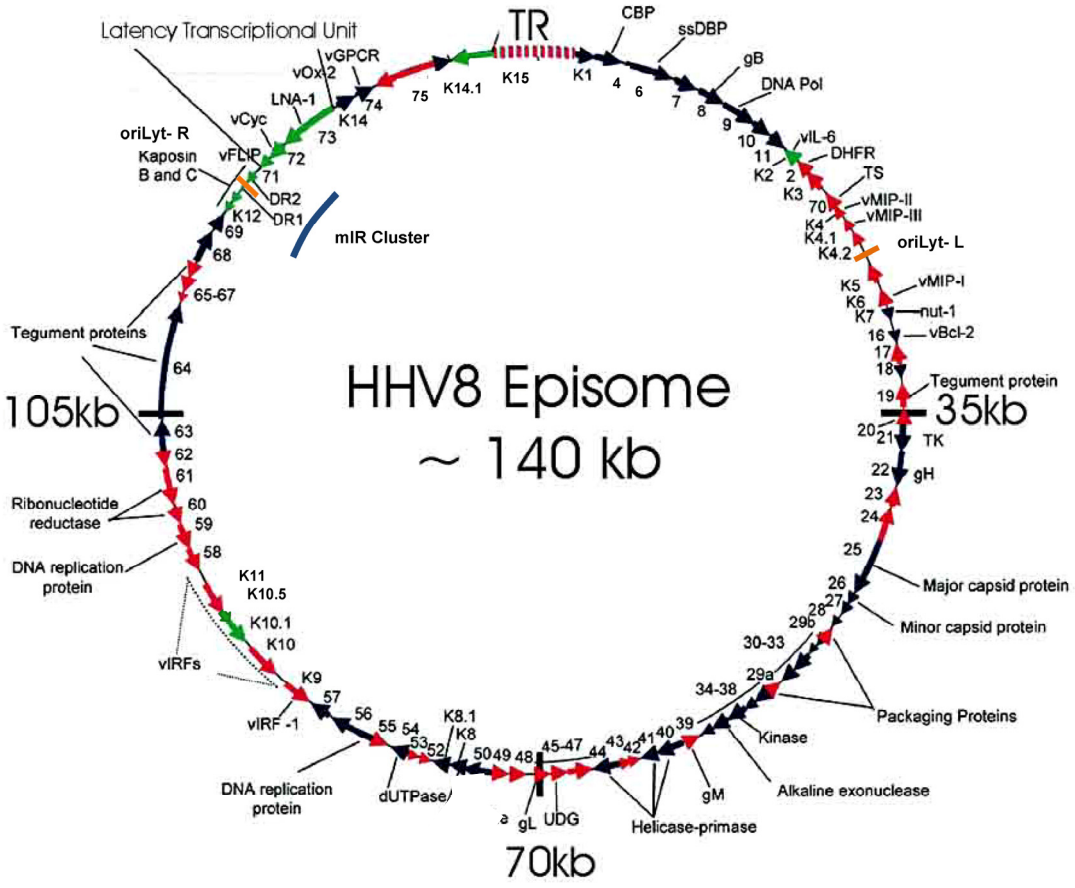

Figure 1-1 The KSHV Genome
The KSHV genome is around 170 kb and consists of a ~140 kb long unique coding region (LUR) flanked by multiple GC rich ~800bp terminal repeats (TR, shown as red boxes). Distance along the LUR is shown in kb. Upon infection, the Kaposi's sarcoma-associated herpesvirus

(KSHV) genome is delivered to the nucleus where it circularises into an episome. The TR regions are used to anchor KSHV episomes to the host chromatin and are therefore essential for latent replication. KSHV also contains two origins of lytic replication (Ori-Lyt-L and Ori-Lyt-R, (orange lines). During the latent programme, a small number of proteins are expressed (green arrows). ORFs encoded on the forward strand i.e. left-to-right are shown in black, while genes encoded on the opposite strand are shown in red. The position of the mIR cluster is shown by the blue line and shown in more detail in Figure 1-5. Adapted from (Cotter and Robertson, 2002).

Since this first characterisation additional ORFs have been described. The divergent regions of the KSHV genome contain more than 20 unique genes, and approximately a quarter of KSHV genes encode several proteins due to differential splicing or alternative promoter usage (Zheng, 2003). Several KSHV ORFs code for genes that have significant homology to cellular genes (Holzerlandt et al., 2002). These genes appear to have been 'pirated' by KSHV in order to manipulate host processes, for example a cyclin D2 homologue, v-cyclin, ensures cell cycle progression (Cesarman et al., 1996) (see section 1.1.12.3). More recently, the KSHV genome has been shown to produce non-coding RNA, including a set of viral micro-RNAs which are expressed during latency (Cai et al., 2005) (Pfeffer et al., 2005) (Samols et al., 2005) (see section 1.1.12.7) and the 1077 bp polyadenylated nuclear RNA (PAN) produced during lytic replication (Conrad and Steitz, 2005) (Sun et al., 1996).

1.1.3.1 KSHV strain diversity

Four major variants of KSHV have been identified by examining the levels of genetic variation between KSHV isolates from various locations around the World (Zong et al., 1999). Large parts of the KSHV genome are highly conserved, however several regions were shown to be highly variable primarily the ORFs K1 and K15. Analysis of the sequence variability of the K1 gene has facilitated the classification of KSHV isolates into the sub-types A, B, C, D and E (Hayward and Zong, 2007). General genome variation between clades is around 3 % however up to 15-30 % variation is seen in K1 at the nucleotide level, particularly in two hypervariable regions (V1 and V2) where as much as 60 % variation is reported (Zong et al., 1999) (Zong et al., 2002). It is not clear what selection pressure is driving this sequence variation. However, epitopes for cytotoxic T-cells (CTLs) are present in the V1 region indicating CTL evasion may contribute to K1 evolution (Stebbing et al., 2003). Also the ability to interact with host intracellular signalling may be involved, as the immune-receptor tyrosine-based activation motif (ITAM)-like sequence in the cytoplasmic region

of K1 shows strong divergence between clades (Hughes and Hughes, 2007). Importantly, variants are stable within an individual host i.e. multiple clones sequenced from a single patient are identical. Sub-type correlates with geographical/ethnic origin of the host. Clades A and C are predominantly isolated from infected individuals in Europe, the United States, Asia and the Middle East, sub-group B strains are found principally in sub-Saharan Africa, while D and E strains are located primarily in south Asia, Australia, the Pacific and Brazilian Amerindians (Zong et al., 1999) (Cook et al., 1999) (Meng et al., 1999) (McGeoch and Davison, 1999) (Biggar et al., 2000). From this information it can be inferred that KSHV is an ancient human virus which arose in Africa. The separation of sub-groups resulted from the isolation and founder effects associated with the several migrations of people from Africa (Hayward, 1999).

Further diversity has been discovered in the region surrounding ORFK15. Variants are known as P (or prototype) or M (or minority) (Poole et al., 1999) and more recently two further variations were seen and designated N and Q (Zong et al., 2002). Only two of the KSHV sub-types, A and C, have these variations that are thought to arise from a recombination event with an unknown progenitor herpesvirus, which occurred after the first two waves of emigration from Africa (Zong et al., 1999).

1.1.4 KSHV virion structure

Herpesvirus architecture is distinct from all other viruses. The linear double stranded DNA genome is packaged as a torus, and encased in an icosahedral capsid composed of 162 capsomeres resulting in a diameter of 115-130 nm (Furlong et al., 1972) (Wu et al., 2000a). Surrounding the nucleocapsid is the tegument, an organised layer composed of various protein species that appears to mirror the shape of the capsid (Zhou et al., 1999). The outer layer of the virus is a lipid bilayer envelope, embedded with viral proteins that are usually glycosylated (Skepper et al., 2001) (see Figure 1-2).

Recent examination of the protein composition of the KSHV virion has revealed that both host and viral proteins are incorporated into the tegument during viral egress (Bechtel et al., 2005b) (Zhu et al., 2005). These proteins include the viral transactivator of lytic replication, RTA, and are thought to be responsible for the

very early events of virus infection. Host proteins found in the virion included the abundant chaperone Hsp90, actin and tubulin. Whether any protein is actively incorporated or simply included during the budding and envelopment process, remains to be determined. This may also apply to the 11 viral messenger RNAs (mRNAs) also shown to be present in the KSHV virion (Bechtel et al., 2005a). However, one incorporated transcript ORF17, encoding viral protease, was detected in the cytosol at lower levels than other un-incorporated controls; indicating a possible selective mechanism of inclusion. At least one of the transcripts, ORF59, was also shown to be translated. Many of the products of these RNAs have anti-apoptotic and immune-modulatory functions that can adapt the host cell environment for infection. The presence of RTA protein and viral mRNA, encoding predominantly lytic products, in the virion could potentially explain the burst of viral lytic genes seen immediately after infection (Krishnan et al., 2004).

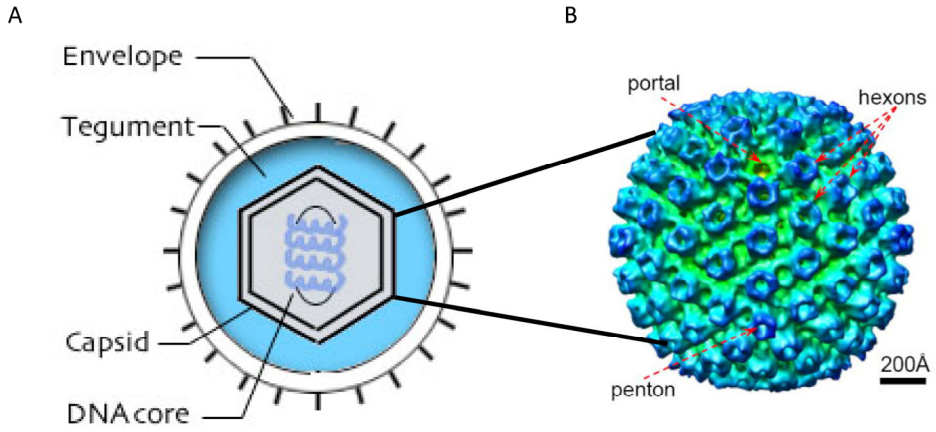

Figure 1-2 Morphology of the KSHV virion
(A) Cartoon of a herpesvirus virion structure. KSHV shares the same morphology and structure as other members of the herpesvirus family. Herpesviruses are surrounded by a lipid membrane (envelope), which is studded with virus glycoproteins (not shown to scale). Within this is a protein layer known as the tegument. The final layer is the capsid, an icosahedral protein shell that contains the virus genome packaged as linear double stranded DNA. Taken from stdgen.northwestern.edu.(B) Radially coloured surface representation of an averaged KSHV capsid showing the characteristic herpesvirus capsomers, including an ‘‘umbilical’’ portal, 11 pentons and 150 hexons. Adapted from (Deng et al., 2008). Bar, 200 Å.

The KSHV capsid was revealed by cryo-electron microscopy (cryoEM) reconstruction to have a T-16 icosahedral structure with 20 triangular faces. The capsomers are composed of hexamers (hexons) and pentamers (pentons) of the major capsid protein (MCP/ORF25). A capsid is comprised of 150 hexons and 12 pentons, connected by 320 triplexes composed of an ORF62 monomer and a ORF26 dimer arranged in a lattice (Figure 1-2 B) (Wu et al., 2000a).

Recently, studies using cryo-electron topography (cryoET) have allowed the study of individual KSHV capsids to obtain structural information that is not averaged over a population (Deng et al., 2008). Comparison of the structures and their scaffold cores have led to a mechanistic model for herpesviral capsid assembly and maturation (see section 1.1.5).

1.1.5 KSHV assembly and maturation

The crucial steps in herpesvirus morphogenesis have been predominantly studied in viruses such as herpes simplex virus (HSV). However, within the family *Herpesviridae* the alpha, beta and gamma subfamilies have similar virion morphology suggesting virion assembly and maturation occurs via comparable mechanisms (Mettenleiter, 2004) (detailed review in (Mettenleiter et al., 2009)). Despite this there is considerable variation between the herpesviruses and further work is required to determine the exact mechanism for each virus that will incorporate the unique genes and their specific functions. Herpesviruses have complicated and highly structured virus particles which include both viral and cellular proteins (see section 1.1.4) (Bechtel et al., 2005b) (Bechtel et al., 2005a) (Zhu et al., 2005). A study of the protein-protein interactions between virion-associated KSHV proteins identified a total of 37 interactions between capsid and tegument proteins and between tegument proteins, as well as between tegument and glycoproteins; providing evidence that the virus particle is constructed through highly specific interactions (Rozen et al., 2008).

The first step in general herpesvirus virion assembly is nucleocapsid formation, an autocatalytic process that occurs in the nucleus. Capsid proteins build around scaffold proteins to form a pro-capsid (Newcomb et al., 1996). The procapsid then undergoes angularisation, catalysed by viral proteases, which results in a capsid with T-16 icosahedral symmetry (Trus et al., 1996). Distinct capsid intermediates can be seen using cryo-EM technology, profiling the mechanism of viral capsid assembly and maturation (Deng et al., 2008). Newly replicated herpesviral DNA is then cleaved and encapsulated through a portal channel, a process similar to that of dsDNA bacteriophage packaging (Newcomb et al., 2001) (Deng et al., 2007).

Herpesvirions complete their morphogenesis via acquisition of tegument and a 'two-step envelopment' process (Skepper et al., 2001). The first step is nuclear egress and is governed by highly conserved proteins; this involves primary envelopment of the capsid via budding through the inner nuclear membrane (Fuchs et al., 2002) (Gonnella et al., 2005). Capsids then gain access to the cytoplasm via budding through the outer nuclear membrane 'de-envelopment'. Processes that occur in the cytoplasm are less well conserved and are complex due to the large number of proteins involved, in addition to a degree of redundancy. Tegumentation occurs via the interaction of viral proteins at two sites, proximal to the capsid and at the future envelopment site, which combine to form a highly ordered network surrounding the capsid (Mettenleiter, 2006). The composition of tegument varies and major changes can occur without significant cost to infectivity. Finally, secondary envelopment through the trans-Golgi network is driven by protein interactions between the two 'subassemblies' of tegument. At this stage glycoproteins are acquired leading to the formation of a mature virus particle, which fuses with the plasma membrane and is released (Turcotte et al., 2005). Herpesvirus virion assembly therefore occurs in both the nucleus and the cytoplasm and consists of two distinct budding and fusion events. The first at the nuclear membrane and the second though the trans-Golgi network to the plasma membrane and out of the cell.

1.1.6 Cell tropism

Gamma-herpesviruses are lymphotropic (Neipel et al., 1998). KSHV DNA has been detected in CD19+ B-cells and is associated with two B-cell lymphoproliferative diseases, PEL and MCD (see section 1.1.8) (Ambroziak et al., 1995) (Harrington et al., 1996) (Mesri et al., 1996) (Campbell et al., 2005) (Dupin et al., 1999). KSHV infection has also been found in peripheral blood monocytes as well as the infiltrating monocytes and endothelial (spindle) cells of KS lesions (Blasig et al., 1997) (Monini et al., 1999) (Boshoff et al., 1995). However, infection of primary or transformed B-cells *in vitro* is very inefficient (Renne et al., 1998) (Blackbourn et al., 2000b) (Bechtel et al., 2003) (Mesri et al., 1996) (Kliche et al., 1998) (Gasperini et al., 2005). The reason for the lack of infectivity of B-cells *in vitro* is not understood as B-cells are the principal KSHV infected cell in peripheral blood. However, a recent study has indicated the requirement of an activated phenotype and the expression of potential co-

receptors such as the dendritic-cell (DC)-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN; CD209) for B-cell infection (Rappocciolo et al., 2008).

Recently, oral epithelial cells have been proposed as a target cell for primary KSHV infection (Duus et al., 2004). Further study using models for keratinocyte differentiation have shown that, like other viruses such as human papillomavirus (HPV), KSHV lytic-gene expression is activated in response to epithelial differentiation and results in the production of infectious virions at the epithelial surface. KSHV DNA and infectious virions have also been found in the saliva indicating saliva exchange as an important route of KSHV transmission (Boldogh et al., 1996) (Pauk et al., 2000) (Vieira et al., 1997) (see section 1.1.7). KSHV lytic reactivation has been linked to B-cell differentiation into plasma cells via the transcription factor XBP-1; if this occurs at the oral mucosal surfaces then KSHV may be shed into saliva and spread to the epithelium (Wilson et al., 2007). This suggests that B-cells are the latent reservoir for KSHV infection *in vivo*, when triggered to differentiate KSHV is produced and infects the oral mucosa and the lymphoid tissue of the Waldeyer's ring; leading to virus shedding into the saliva (Longworth and Laimins, 2004) (Johnson et al., 2005a) (Pauk et al., 2000) (Chagas et al., 2006). Most cases of primary KSHV infection are asymptomatic and recognised only by the presence of antibody to viral proteins. The initial infection of children and immunocompromised adults however, often leads to a burst of viraemia, which can cause a febrile illness and lymphadenopathy (Wang et al., 2001b) (Kasolo et al., 1997).

In vitro, KSHV infects more than 20 cell types of both human and non-human origin, including those known to be infected *in vivo*, and a range of adherent cell lines including human fibroblasts, 293 cells, microvascular endothelial cells (MVECs), human umbilical vein endothelial cells (HUVECs), rat endothelial cells and mouse fibroblasts (Blackbourn et al., 2000b) (Renne et al., 1998) (Bechtel et al., 2003) (Flore et al., 1998). Infection of these cells predominantly leads to latency, and many cell lines can be cultured for a significant period of time (Vieira et al., 2001) (Vieira and O'Hearn, 2004). In general, KSHV in established cell lines responds only weakly to reactivation stimuli. Therefore, these cell lines cannot be used to produce high titre KSHV virus. However, addition of the viral

regulator of transcription activation (RTA) does lead to lytic replication in many of these cell lines, indicating the block to reactivation in these cell lines is at the induction of expression of RTA (Bechtel et al., 2003). Of the many cell types infected MVECs have proven the most useful as an experimental system. This is due to the fact that MVECs are thought to be a precursor cell type for KS lesions, and they are frequently latently infected with a small spontaneous lytic population, a situation similar to *in vivo* conditions (Ciufo et al., 2001).

Despite the potential of the MVEC system and PEL cell lines (see section 1.1.8.2), the study of lytic replication, immune responses and pathology are still impeded by the lack of lytic replication *in vitro* and the absence of an animal model for KSHV infection and its associated diseases (Renne et al., 1998) (Mutlu et al., 2007) (reviewed in (Chang et al., 2009) (Parsons et al., 2006)). A recent study has shown successful infection of a new world primate, the common marmoset, and has identified persistent infection as well as possibly KS-like disease (Chang et al., 2009). This will facilitate further investigation into host-viral interactions in the context of an intact host immunity.

1.1.7 Epidemiology and transmission

KSHV prevalence can be divided by geographic location and by HIV risk groups (reviewed in (Bagni and Whitby, 2009)). In regions such as North America, North Europe and Asia the prevalence of KSHV in the population is low, between 1- 7 % (Gao et al., 1996b) (Pellett et al., 2003) (Simpson et al., 1996). Intermediate levels of KSHV prevalence of around 5-25 % are seen in the Mediterranean, Middle East and the Caribbean (Calabro et al., 1998) (Serraino et al., 2001). The highest levels of KSHV seropositivity are seen in sub-Saharan Africa and the Amazon basin where greater than 50 % of the population are thought to be infected (Sitas and Newton, 2001) (Engels et al., 2000). The incidence of Kaposi Sarcoma (KS) is correlated with KSHV seroprevalence. The transmission and risk factors for KSHV infection are not completely understood, however the different epidemiology in these regions indicates two major routes of infection, sexual and non-sexual contact.

In low-prevalence areas, including Western Europe and the USA, sexual transmission is thought to be the predominant route of transmission. In these

regions children, before sexual maturity, are seronegative while the risk of conversion increases with age. KSHV is frequent in homosexual men and peaked during the AIDS epidemic, where seroprevalence was reported to be between 25-60 % (Simpson et al., 1996) (Chatlynne et al., 1998) (Dukers et al., 2000). A clear link between the number of sexual partners and risk of KSHV indicates transmission via sexual contact in this group (Martin et al., 1998) (Martro et al., 2007). Interestingly, in low-prevalence areas seroprevalence in women is lower than in men, highlighting heterosexual practices as an inefficient route of KSHV transmission (Kedes et al., 1997a) (Smith et al., 1999) (Engels et al., 2007) (de Sanjose et al., 2009). Parenteral transmission appears to play a small part in KSHV epidemiology as the prevalence is much lower than other viruses that are known to be transmitted via this route, HIV, HBV, HCV (Renwick et al., 2002) (Atkinson et al., 2003) (Zavitsanou et al., 2010). Cases of KSHV infection in the recipients of blood transfusions have been reported, suggesting donors should be screened (Hladik et al., 2006). However, due to limited resources depleting blood of leukocytes or storing the blood before use would reduce the risk of transmission. An increased risk of developing KS is associated with transplantation, this is primarily due to reactivation of KSHV in immune-suppressed KSHV positive recipients, but can be caused by transmission from seropositive donor to seronegative recipient (Luppi et al., 2000) (Cattani et al., 2001) (Barozzi et al., 2003) (Frances et al., 2009).

In the high-prevalence areas such as sub-Saharan Africa and the Amazon basin, the epidemiology is starkly different with infection occurring more often at childhood, and with equal seroprevalence amongst adult men and women (Sarmati, 2004) (Klaskala et al., 2005). The high numbers of childhood infection highlights a non-sexual route of transmission, presumably through salivary exchange as there is no evidence for transmission through breast milk and congenital infection occurs rarely (Brayfield et al., 2004) (Mantina et al., 2001). In children KSHV is therefore acquired horizontally via intra-familial contact (Plancoulaine et al., 2000) (Dedicoat et al., 2004) (Malope et al., 2007). It was also shown that HIV co-infection leads to a highly significant increased risk of childhood KSHV infection (Minhas et al., 2008). Seroprevalence rises gradually after puberty to mid-adulthood, reflective of poor heterosexual transmission (Baeten et al., 2002) (Eltom et al., 2002). KSHV is however found at low levels

in genital secretions suggesting that heterosexual transmission is possible although highly debated (Calabro et al., 1999) (Howard et al., 1997) (Diamond et al., 1997) (Taylor et al., 2004).

The majority of herpesviruses are transmitted via saliva and are acquired early in childhood leading to a large proportion of seropostive adults. KSHV virions are most readily detected in the saliva indicating a role in transmission (Pauk et al., 2000) (Vieira et al., 1997). Despite this, the prevalence of KSHV in some areas is far lower than other herpesviruses EBV, HCMV, HHV6 and 7, which are almost ubiquitous in the adult human population (reviewed in (Iscovich et al., 2000)). An exception is in South America among the Amerindians where KSHV is hyper-endemic, with 79 % of the population infected likely through oral-transmission routes (de Souza et al., 2007). Non-Amerindians living in the same region displayed only 6 % seropositivity indicating that the factors contributing to KSHV transmission in this population are more associated with behaviour or genetic susceptibility to infection rather than environmental factors. However, the vast geographical differences in KSHV prevalence must be in part due to environmental co-factors. Studies in Italy and northern Sweden suggest that biting insects may play a role in transmission, as insect bites may be licked in order to sooth pain, exposing the wound to infected saliva (Coluzzi et al., 2002) (Ascoli et al., 2006). A Ugandan study reported the source of water as a risk factor for KSHV infection among other socioeconomic factors (Mbulaiteye et al., 2005). Finally, a screen of naturally occurring compounds for their ability to induce KSHV reactivation led to the identification of plant products used in traditional medicines by some cultures; thus exposure may increase shedding (Whitby et al., 2007). Together these findings indicate that the transmission of KSHV is dependent on a combination of both environmental and genetic factors.

1.1.8 KSHV-associated disease

Diseases associated with KSHV infection occur predominantly in the immune-suppressed and is important to remember that KSHV infection most frequently occurs without causing disease.

1.1.8.1 Kaposi Sarcoma (KS)

Kaposi Sarcoma (KS) is a highly vascular, non-classical tumour, composed predominantly of KSHV-infected cells; the spindle cells (Boshoff et al., 1995) (Ensoli et al., 2001a) (Schwartz et al., 2008) (Gessain and Duprez, 2005). These spindle cells are named according to their morphology, can be oligoclonal and are of endothelial origin but poorly differentiated (Gill et al., 1998) (Judde et al., 2000) (Duprez et al., 2007). KS spindle cells express molecular markers of two closely related cell types; including CD31 and CD36 present on blood vessel endothelial cells (BECs), as well as markers of lymphatic endothelial cells (LECs), VEGF-R3 and podoplanin, making it difficult to identify the precursor cell type of KS (Regezi et al., 1993) (Dupin et al., 1999) (Beckstead et al., 1985) (Staskus et al., 1997) (Weninger et al., 1999). More recently, studies have reported that the spindle cells of KS-lesions have a similar gene expression profile to LECs (Wang et al., 2004a). In addition latent KSHV infection of BECs has been shown to cause reprogramming and the induction of lymphatic lineage-specific genes (Carroll et al., 2004) (Hong et al., 2004b) (Hansen et al., 2010).

KS is also characterised by the presence of infiltrating macrophages, plasma cells and T-lymphocytes. Lesions are interspersed with slit-like neovascular spaces, which are not lined by smooth muscle or pericytes and are therefore susceptible to leakage (Dupin et al., 1999). Clinically, KS presents as reddish brown lesions of the dermis, found cutaneously, mucosally or viscerally. The colour is due to the breakdown of erythrocytes that enter the lesion via the weak neo-vasculature. KS development can be staged into 6 overlapping forms: patch, plaque, nodular, lymphadenopathic, infiltrative and florid (Wen and Damania, 2009). As the lesion progresses, the ‘driving’ KSHV infected spindle cells gradually predominate, indicating a growth/survival advantage (Boshoff et al., 1995) (Staskus et al., 1997) (Dupin et al., 1999). KS derived cells do not display all the traits of transformed cells. In general, they lack aneuploidy, cannot grow in soft agar and when injected into nude mice do not form tumours, but survive briefly to encourage neovascularisation (Salahuddin et al., 1988). These cells also continue to require exogenous factors for growth (Ensoli and Sturzl, 1998) (Marchio et al., 1999). Intriguingly, many viral lytic pro-inflammatory and angiogenic gene products may be involved in the paracrine

stimulation of sarcomagenesis (reviewed in (Hayward, 2003)). This highlights the importance of a small spontaneous lytic population of KSHV infected cells in KS (Parravicini et al., 2000) (Katano et al., 2000).

KSHV was identified from KS biopsies in 1994; since then the evidence for its involvement in KS development has been obtained (Chang et al., 1994). Firstly, KSHV viral DNA and antigens have been found in nearly all KS spindle cells but are absent in surrounding healthy tissues (Boshoff et al., 1995) (Huang et al., 1995) (Moore and Chang, 1995) (Dupin et al., 1999). Secondly, KSHV infection occurs prior to onset of AIDS-KS and viral load can be used to predict risk of KS development (Moore et al., 1996b) (Whitby et al., 1995) (Renwick et al., 1998). Thirdly, prophylactic treatment with anti-herpetics (e.g. ganciclovir) can reduce the risk of developing KS (Martin et al., 1999) (Robles et al., 1999) (Casper and Wald, 2007). In addition the KSHV present in advanced lesions is monoclonal indicating that infection occurs before sarcomagenesis (Judde et al., 2000). Finally, the expression of the viral homolog of a G-protein coupled receptor (GPCR) in mouse endothelial cells, *in vivo*, causes angioproliferative tumours reminiscent of KS, highlighting the oncogenic potential of KSHV encoded genes (Montaner et al., 2003). The role of KSHV in KS development is complex and involves both latent and lytic genes, many of which are pirated versions of cellular genes. The oncogenic potential of latently encoded genes can lead to a cellular growth advantage, which in combination with paracrine signals expressed during the lytic cycle can cause hyperproliferation and transformation (see sections 1.1.12 and 1.1.13 (reviewed in (Schulz, 2006) (Ganem, 2006) (Martin and Gutkind, 2008) (Ganem, 2010)).

Lytic gene involvement in KSHV-associated disease pathogenesis is further complicated by the cytotoxicity of lytic replication. Therefore, if a gene is to contribute to disease progression, for example via a paracrine mechanism such as vGPCR upregulation of VEGF, then substantial lytic replication would be required. This is in fact what is seen as higher viral loads are detected in those with disease (Campbell et al., 2000) (Quinlivan et al., 2002). It is also necessary to circumvent the shut off of host gene expression mediated by another KSHV lytic gene product SOX, although this is not thought to be complete (see section 1.1.13).

On the basis of epidemiology KS can be divided into four distinct forms: classic/sporadic, endemic/African, AIDS-related/epidemic and post-transplant/iatrogenic (reviewed in (Schwartz et al., 2008)). Moritz Kaposi first identified classic KS in 1872. This form occurs predominantly in the elderly male population of the Mediterranean, Eastern Europe and Arab nations as well as those of Jewish ancestory. This indolent form usually presents as lesions in the lower and upper extremities without lymph node and internal organ involvement (Iscovich et al., 2000). Endemic KS is also known as African KS because it is commonly seen in Central and East African countries. This type of KS can be indolent or aggressive (lymphadenopathic), although the latter is more often found in children and has high fatality rates. The most frequent and aggressive form of KS is AIDS-related and can include lymph node and visceral spreading. High HIV viral load has been associated with increased KS risk, while the number of KS cases reported after the initiation of highly active antiretroviral therapy (HAART) decreased (Gates and Kaplan, 2002b) (Gates and Kaplan, 2002a). KS regression is also seen after HAART (Cattelan et al., 2001). Due to the strong association with HIV infection and AIDS outlined above, KS was identified as an AIDS-defining illness in the mid 1980s (Mbulaiteye et al., 2003). The role HIV infection plays in KS development is unclear, the HIV tat protein has been shown to encourage KS development as well as inducing KSHV lytic replication, possibly disseminating virus to endothelial cells, again increasing risk of KS (Harrington et al., 1997) (Varthakavi et al., 2002). HIV-associated immunodeficiency may also play a key role, as this inhibits the host's ability to control KSHV lytic replication, again increasing the number of endothelial cells infected. This assumption is supported by the final type of KS, post-transplant, which is also associated with immune-suppression, although drug-induced, and can originate from KSHV infected donor cells (Barozzi et al., 2003). This link to immunodeficiency also highlights another important aspect of KS, in that KSHV is necessary but not sufficient for KS development and cofactors are also required (Calabro et al., 1998). The fact that the risk of KS is low even in areas of high prevalence illustrates this point. For example, most of the KS cases reported in Africa are in East and Central regions despite high seroprevalence in other areas (Cook-Mozaffari et al., 1998).

1.1.8.2 Primary Effusion Lymphoma (PEL)

PEL is a rare and aggressive form of non-Hodgkin lymphoma (NHL) associated with KSHV infection, and found predominantly in late stage AIDS patients (Cesarman et al., 1995) (Nador et al., 1996) (Knowles, 2003) (reviewed in (Carbone et al., 2010)). The disease is primarily characterised by a lymphomatous effusion in the serous cavities including the pericardium, pleurum and peritoneum, however lymph node and solid organ involvement is sometimes seen (Chadburn et al., 2004) (Carbone et al., 2005). Although most cases are observed in the AIDS-related immune-deficient environment, AIDS-unrelated cases have been reported and are clinically identical but tend to develop later in life (Carbone et al., 1996). PEL has also been found in association with iatrogenic immune-suppression linked to solid organ transplant (Kapelushnik et al., 2001) (Boulanger et al., 2008).

The origin of PEL cells was difficult to determine due to the absence of classical B-cell markers such as CD19, however analysis of immunoglobulin (Ig) rearrangements confirmed not only a B-cell origin but also that PEL is a clonal malignancy, suggesting latent infection occurs before PEL develops (Nador et al., 1996). PEL predominantly have somatically hypermutated Ig genes indicating a post-germinal centre origin (Matolcsy et al., 1998) (Arvanitakis et al., 1996) (see section 1.4.2). PEL cells do not have Ig on their surface or produce Ig for secretion, partly due to the absence of B-cell specific members of the POU family of transcription factors, Oct-2 and OCA-B (Di Bartolo et al., 2009). PEL do express B-lymphocyte induced maturation protein-1 (BLIMP-1) and the cleaved form of activating transcription factor-6 (ATF-6) but express the inactive form of the transcription factor, X-box binding protein-1 (XBP-1) (see section 1.4.4). This transcription factor profile indicates that PEL represent a pre-terminal, plasmablastic stage of B-cell differentiation, which has been previously indicated by gene expression profiling (Wilson et al., 2007) (Jenner et al., 2003) (Klein et al., 2003) (Carbone et al., 2001) (see section 1.4.5). PEL cells express syndecan-1 (CD138), a marker of pre B-cells and plasma cells which is not expressed on any other lymphomatous effusion and can therefore be used for clinical diagnosis (Gaidano et al., 1997).

Evidence for the contributing role of KSHV in PEL development is attributed to the identification of approximately 2-200 viral DNA episomes in all PEL cells (Cesarman et al., 1995) (Dupin et al., 1999). EBV is also reported in 70 % of PEL cases and may be involved in tumour progression after the germinal centre reaction, although little or no expression of EBV transforming genes are detected in PEL (Horenstein et al., 1997) (Szekely et al., 1998) (Gaidano et al., 2000). Comparisons of the gene expression profiles of PEL with KSHV negative lymphomas identify many differentially expressed genes, while comparisons of EBV positive and negative PEL have shown only subtle differences (Hamoudi et al., 2004) (Klein et al., 2003) (Fan et al., 2005). This work identifies the dominant role of KSHV in PEL. PEL cell survival has been shown to depend on the expression of KSHV latent genes, again highlighting the association between KSHV infection and this disease (Guasparri et al., 2004) (Godfrey et al., 2005) (Wies et al., 2008). Despite this clear correlation PEL occurs very rarely even in endemic KSHV areas. Cases of KSHV negative, EBV negative PEL have been reported and can be associated with HCV and liver cirrhosis (Kobayashi et al., 2007) (Hara et al., 2001).

KSHV is therefore a risk factor for PEL development and like other DNA tumour viruses, further transforming events must occur. In PEL, the BCL-6 gene is often mutated and chromosome abnormalities are also seen such as trisomy 7 and 12, however; no abnormality is common to all cases indicating the importance of KSHV infection (Gaidano et al., 1999) (Gaidano et al., 2000) (Wilson et al., 2002). The immune-system also has a key role in controlling this disease as HIV patients on HAART have a better clinical prognosis, and efficient tumour formation is observed when PEL cells are supplied to nude mice (Boulanger et al., 2005) (Picchio et al., 1997).

How KSHV directly contributes to this B-cell malignancy is not completely understood, and as yet infection of primary B-cells is inefficient and does not lead to transformation (see sections 1.1.12 and 1.1.13) (reviewed in (Schulz, 2006)) (Rappocciolo et al., 2008). However, PEL cells readily grow in culture providing the first and most robust system for studying KSHV infection (Arvanitakis et al., 1996) (Gaidano et al., 1996) (Renne et al., 1996a) (Renne et al., 1996b) (Boshoff et al., 1998). In this system KSHV is predominantly latent

(see section 1.1.12) with less than 1 % of cells entering the lytic cycle, unless induced to reactivate, and has therefore been fundamental in facilitating the study of KSHV (Miller et al., 1997) (Shaw et al., 2000) (Parravicini et al., 2000) (Katano et al., 2000).

1.1.8.3 Multicentric Castleman's Disease (MCD)

MCD is an unusual lymphoproliferative disorder of plasmablast cells characterised by lymphadenopathy, splenomegaly, fever and weight loss (reviewed in (Waterston and Bower, 2004) (Stebbing et al., 2008)). The plasma cell variant of MCD occurs predominantly in AIDS patients and is frequently linked to KSHV infection (Soulier et al., 1995) (Dupin et al., 2000). KSHV DNA is detected predominantly in plasmablasts located in the mantle zone regions of germinal centres in all HIV-associated cases of MCD and between 40-50 % of those unrelated to HIV infection (Soulier et al., 1995) (Luppi et al., 1996) (Dupin et al., 1999) (Parravicini et al., 2000). HAART in combination with chemotherapy has lengthened the life expectancy of MCD patients, probably more indicative of fewer opportunistic infections rather than an effect on MCD as chemotherapy is required to prevent reoccurrence of MCD (Aaron et al., 2002).

The KSHV positive cells are lambda light chain restricted, for as yet unknown reasons, but are polyclonal as monitored by the Ig rearrangements as well as KSHV TR regions (Du et al., 2001) (Judde et al., 2000). KSHV positive plasmablasts are not co-infected with EBV, and probably result from KSHV infection of naive B-cells (Murray et al., 1995) (Dupin et al., 1999) (Du et al., 2001) (Chadburn et al., 2008). KSHV viral load and cytokines, for example vIL-6, have also been reported to contribute to the disease state, MCD (Oksenhendler et al., 2000). A proportion of KSHV infected cells of MCD undergo lytic replication and may be responsible for the pathology and severity of the disease (Parravicini et al., 2000) (Katano et al., 2000) (reviewed in (Schulz, 2006)). The use of anti-virals has led to a reduced frequency of acute symptoms in MCD patients again linking KSHV to the pathogenesis of this disease (Casper et al., 2004). When used in combination with rituximab treatment, which depletes CD20 B-cells, a possible increase in remission of MCD may be seen (Gerard et al., 2007).

1.1.8.4 KSHV-associated disease treatment

The predominantly latent KSHV infection of KS and PEL tumour cells prevents the use of anti-herpetics that target lytic replication (Boshoff et al., 1995) (Dupin et al., 1999) (reviewed in (Klass and Offermann, 2005)). In contrast, the KSHV lytic population of the cells in MCD is far higher, and treatment with anti-virals has proved successful (Parravicini et al., 2000) (Casper et al., 2004). Therefore, as described with KS, inducing lytic replication in PEL cells could make anti-herpetic drugs, such as ganciclovir, useful therapeutic agents in treating this neoplasm (Martin et al., 1999). The inhibition of lytic replication may also be fundamental in reducing the risk of disease. Lytic replication in infected B-cells is thought to be required for transmission to endothelial cells, which may initiate KS development (Whitby et al., 1995) (Song et al., 2004). Since the pathogenesis associated with KSHV-related disease, especially in KS, has been linked to lytic gene products, this may also be challenged by a therapy to inhibit lytic replication (Montaner et al., 2003). The potential of exploiting the lytic cycle as a therapeutic target indicates the importance of indentifying the stimuli that cause KSHV reactivation. A better comprehension of host-virus interactions may lead to new strategies for controlling and perhaps eliminating the KSHV latent infection associated with malignancies.

A drug for possible use in the treatment of PEL is valproic acid (or valproate), a histone deacetylase inhibitor commonly used in the treatment of seizures and other neurological disorders. Valproate can reactivate latent KSHV at concentrations similar to plasma concentration after chronic oral treatment (Shaw et al., 2000). Used in combination with ganciclovir, valproate can block KSHV lytic replication and induce apoptosis of PEL cells *in vitro* (Klass et al., 2005). The clinical use of this strategy has also been demonstrated in a patient suffering from post-transplant lymphoproliferative disease, a B-cell neoplasm associated with EBV. Arginine-butyrate was used to induce EBV lytic replication, which was then blocked by the administration of ganciclovir; resulting in substantial tumour necrosis (Mentzer et al., 1998). Another potential therapy is the use of the proteasome inhibitor, Bortezomib (valcade). Bortezomib is an anti-tumour drug shown to be effective against many hematologic malignancies and has been approved for the treatment of multiple myeloma (Hideshima et al., 2001). By inhibiting the degradation of proteins by

the proteasome bortezomib increases the cellular levels of many proteins; modulating the functions of the pathways dependent on these proteins. For example, the inhibitor of NF-κB activity, IκB is no longer degraded causing NF-κB inhibition. Accumulation of protein also induces ER stress and the UPR however, bortezomib interferes with the normal processing of XBP-1 by IRE-1; see section 1.3 (Lee et al., 2003a). Recently, it has been demonstrated that bortezomib has an anti-tumour activity on PEL (An et al., 2004). Its activity here could possibly be due to the stabilisation of XBP-1u which has been shown to induce limited KSHV reactivation (Wilson et al., 2007).

1.1.9 KSHV life cycle

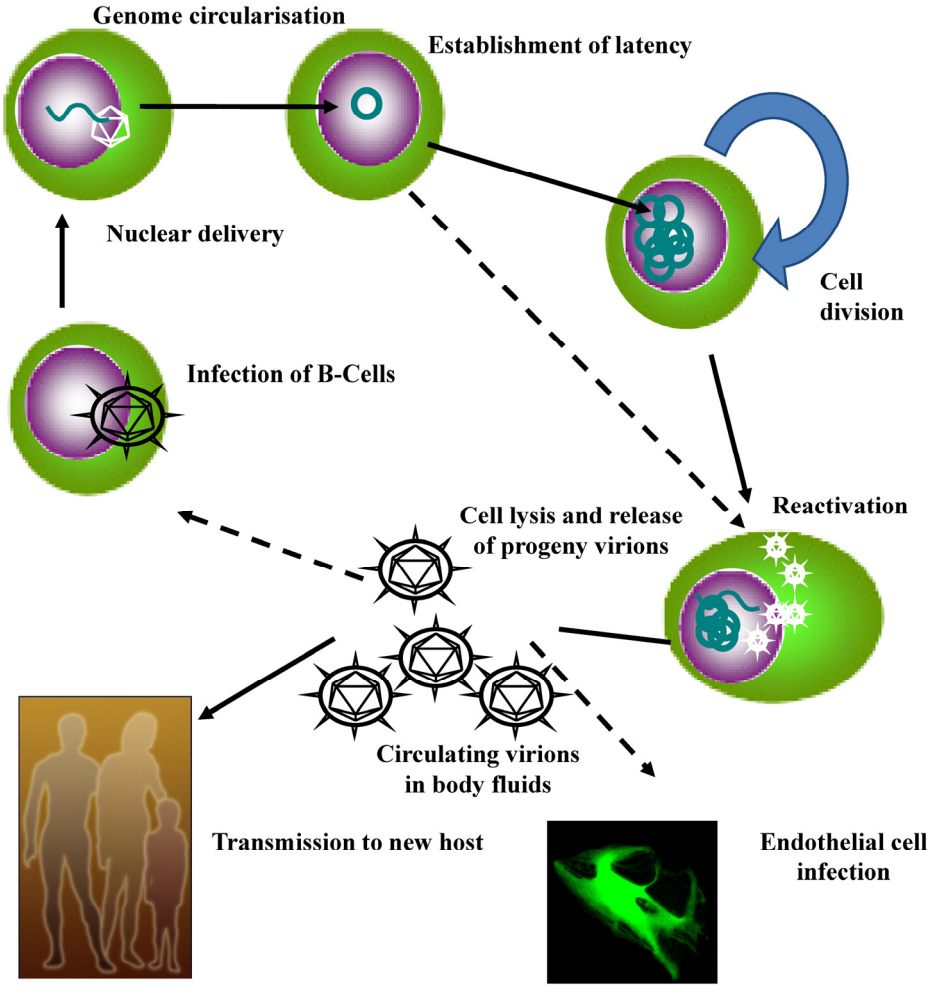

Figure 1-3 General life cycle of KSHV
Upon entering a cell the viral genome forms an episome. Herpesviruses can enter one of two pathways of replication; latent replication, during which the virus genome copied by host DNA replication machinery during the cell cycle, or lytic replication, during which the genome is copied by virus-encoded DNA replication proteins and packaged into progeny virions. Herpesviruses can also reactivate from latency to enter lytic replication, although the

mechanism by which this occurs is poorly understood. Virus replication results in cell lysis and the progeny virions are able to infect more cells or be transmitted to a new host.

KSHV adopts the typical life cycle of a herpesvirus depicted in Figure 1-3. Upon infection, the genome is delivered to the nucleus where it is maintained as a circular episome. KSHV, like all other herpesviruses, can enter one of two phases; either the pathway of latent infection or lytic infection in order to produce progeny virions. During latency the majority of the genome is silenced, and selective promoter regions, for example ORF50, are heavily methylated potentially to maintain latency (Chen et al., 2001). Reactivation, the switch from latency to lytic replication, can be induced by many exogenous signals. However, it is those signals that are relevant to *in vivo* infections that are critical in understanding how the virus and host interact (see section 1.1.14). Lytic replication is the ordered expression of the whole genome in temporally regulated transcriptional cascade (Jenner et al., 2001). Genes are grouped according to their timing of expression. Finally, linear DNA genomes are packaged into nascent viral capsids, tegument is acquired just prior to budding, an event which provides the envelope of host-derived membrane. New virions are released in a lytic burst and can go onto infect other cells within a host or transmit to a new host (Figure 1-3).

1.1.10 Virus Entry

KSHV entry is a multi-stage, complex process recently reviewed by Chandran (Chandran, 2009). The initial attachment of KSHV to the cell surface is mediated by the interaction of viral envelope glycoproteins gB, K8.1, gH and KSHV complement control protein (KCP) with the cellular glycosaminoglycan, heparin sulphate, on the host plasma membrane (Akula et al., 2001a) (Wang et al., 2001a) (Birkmann et al., 2001) (Hahn et al., 2009) (Spiller et al., 2006). This mechanism of concentrating virus at the cell surface, prior to entry, increases infection efficiency and is employed by many herpesviruses including HSV and EBV (O'Donnell and Shukla, 2008) (Tanner et al., 1987). However, the fact that infectivity is not completely blocked by the addition of heparin sulphate binding competitors suggests that this adsorption step is not essential (Akula et al., 2001b) (Luna et al., 2004).

The KSHV envelope embedded glycoproteins; gB, gH and gL have been shown to mediate virus entry in transient expression studies (Pertel, 2002). Glycoprotein, gB, has been shown to bind to integrin α3β1 via an arginine-glycine-aspartate (RGD) integrin binding motif (Akula et al., 2002). However blocking this interaction with antibodies to α3β1 did not abolish infectivity, suggesting that integrins are not the only host receptor involved in KSHV entry (Akula et al., 2002) (Inoue et al., 2003). Also, different combinations of integrins are expressed by different cell-types indicating a cell-type dependent receptor usage. Other studies have also implicated αVβ3 and αVβ3 integrins and the binding of gH/gL to syndecans in adherent cell infection (Garrigues et al., 2008) (Hahn et al., 2009).

A cDNA screen for cellular receptors that facilitate KSHV entry to an otherwise non-permissive cell, revealed the cysteine/glutamate transporter (xCT), which is known to form a cell surface complex that includes CD98 (Kaleeba and Berger, 2006) (Wagner et al., 2001). CD98 is multifunctional molecule identified in association with integrin α3 as fusion regulation protein 1 (FRP-1) (Deves and Boyd, 2000) (Ohgimoto et al., 1995). Studies in HMVEC-d demonstrate that KSHV infection induced the formation of a multi-molecular complex containing α3β1, αVβ3 and αVβ3 integrins and CD98/xCT (Veettil et al., 2008). However, xCT mRNA was not detected in non-activated primary B-cells a known target of KSHV (Kaleeba and Berger, 2006) and the role of integrins in non-adherent cell infection is not clear. KSHV therefore may interact with one set of receptors in adherent cells and may use an alternative receptor(s) in non-adherent cells. Indeed, for the infection of human primary myeloid DCs, macrophages, and B cells, KSHV has been shown to use DC-SIGN, as a receptor (Rappocciolo et al., 2008). Receptor engagement instigates membrane fusion, delivering nucleocapsid to the cytoplasm. This occurs either at the plasma membrane or endosomal membranes in a cell-type dependent way (Akula et al., 2003) (Raghu et al., 2009).

Importantly, virus-cell binding has been shown to activate existing integrin-associated host cell signalling pathways, to augment the cellular environment so that it supports establishment of infection. Pathways include focal adhesion kinase (FAK), Src, phosphatidylinositol 3-kinase (PI3-K), protein kinase C-ζ

(PKC), Rho-GTPases, mitogen-activated protein kinase (MEK), extracellular signal-regulated kinase (ERK1/2), and nuclear factor kappa B (NF-κB)., which are required for the internalization of viral DNA, modulation of cytoskeleton, transport of capsid the microtubules using dynein motors (reviewed in (Lyman and Enquist, 2009)), nuclear delivery of viral DNA as well as the initiation of viral and host gene expression (Akula et al., 2002) (Naranatt et al., 2003) (Naranatt et al., 2005) (Raghu et al., 2007) (Sadagopan et al., 2007) (Sharma-Walia et al., 2005) (Veettil et al., 2006) (Pan et al., 2006). Activation of cell signalling is independent of viral replication as UV-irradiated inactive virus also induces the same responses.

The viral capsids of herpesviruses are too large to enter the nucleus via the nuclear pore complex (NPC). Genomic DNA is therefore actively injected into the nucleus from the nucleocapsid docked to the cytosolic face of the NPC, this is dependent on the presence of importin-β (reviewed in (Greber and Fassati, 2003)). Following delivery the linear genome is circularised, a process thought to depend on host enzymes. Once this is achieved the virus can enter either a latent or lytic transcriptional program.

1.1.11 Establishing Latency

Latency is a feature of all herpesviruses which allows persistent infection of the host. During latency the expression of viral genes from the viral episome tethered to host chromatin is restricted. This limits the number of viral protein epitopes presented to the immune system, allowing the virus to avoid detection. How the virus is directed into latency rather than the lytic gene expression programme is intensely studied. In alpha-herpesviruses this depends on the cellular environment encountered upon infection. HSV-1 infection of oral mucosal epithelium results in lytic replication, however infection of sensory neurons leads to HSV-1 latency (Bastian et al., 1972). This strongly suggests that host cell factors actively influence the establishment of latent or lytic infection.

Latency has been identified as the ‘default’ pathway following failed lytic cycle initiation i.e. immediate early gene expression is blocked (Samaniego et al., 1998). In the case of KSHV, the expression of a number of lytic transcripts and

proteins occurs transiently in the first 12 hours of infection, known as the abortive lytic gene expression programme, before the latency programme succeeds at 24 hours (Krishnan et al., 2004). This intermediate stage, does not represent full lytic cycle as only 29 lytic products were detected, including members from all stages, early to late. The presence of some of these gene products can be partly explained by the discovery that 11 virally encoded lytic mRNAs are incorporated into the tegument of newly made virions (see section 1.1.4) (Bechtel et al., 2005a). RTA, the lytic switch protein was also shown, by one study, to be incorporated into the virus particle, possibly at low levels (Bechtel et al., 2005b). However, the presence of RTA should induce the full lytic cycle not the partial and disorganised lytic cycle observed (Sun et al., 1999) (West and Wood, 2003). It is possible that the viral gene expression seen may relate to the incomplete maturation and histone association of virion DNA (Stedman et al., 2004). Despite the lack of mechanism for the restricted lytic gene expression prior to latency it is thought that the immune-modulatory and anti-apoptotic functions of the genes expressed are required for the progression of infection (Krishnan et al., 2004). Whether the concurrent expression of lytic and latent transcripts transiently occurs in the de novo infection of all cell types is yet to be determined.

1.1.12 Latency

During latency, the viral episome is maintained through successive generations but no viral progeny are produced. The majority of information on KSHV latent gene expression comes from studies with PEL cell lines. This is due to the fact that these cells stably maintain the viral episome and latent viral transcripts are present in all cells. However, the KSHV latent expression profile observed in these cells may not represent all possible forms of latency; as indicated by the viral interferon regulatory factor – 3 (vIRF3/LANA2), which is detected in PEL cells but is absent from latently infected KS spindle cells (Rivas et al., 2001). In general, despite the potential of KSHV to have various latency programmes similar to the closely related EBV, the viral gene products associated with KSHV latency in PEL have been confirmed in other latency models. The similarity in the gene expression profiles of KSHV-associated diseases KS, PEL and MCD also indicate a predominant latency programme (Schulz, 2006). These genes are important for the establishment and maintenance of latency and also have

roles in the tumourigenesis associated with KSHV (reviewed in (Moore and Chang, 2003)).

1.1.12.1 KSHV latency-associated region

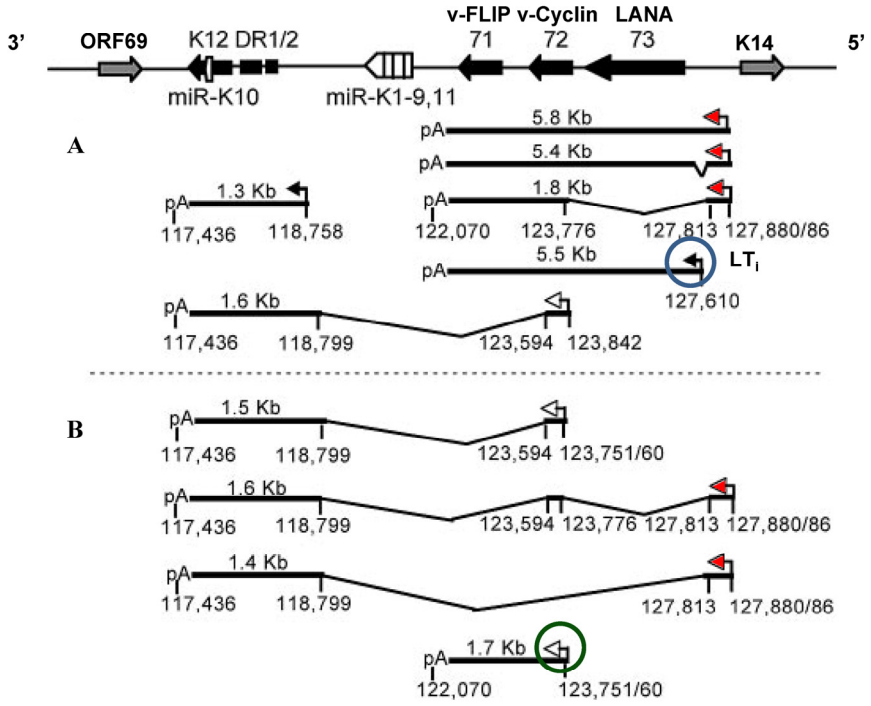

Figure 1-4 Transcription of the latency-associated region of the KSHV genome
The KSHV latency-associated region encodes four proteins, indicated by black boxes, as well as 11 miRNAs (white boxes) and is flanked by lytic genes (grey boxes). (A) Previously reported promoters, splice sites, and poly(A) addition sites are indicated. Lytic promoters are indicated by black arrows and latent promoters by white/red arrows. Novel KSHV transcripts identified in this report are shown in (B). Sequence coordinates are derived from the KSHV genome sequence obtained from BC-1 cells (GenBank accession number NC_003409). Adapted from (Cai and Cullen, 2006).

During latency, KSHV gene expression is restricted to the latency-associated region located between ORFK12 and ORF74 (Figure 1-4). This region encodes four genes as well as the set of viral microRNAs (miRNA) in the same orientation, indicating their production from one primary precursor miRNA (Samols et al., 2005). Within this region at least two promoters are active, leading to the production of a group of transcripts, latency-associated transcripts (LATs) (Figure 1-4) (Talbot et al., 1999) (Pearce et al., 2005) (Cai and Cullen, 2006). The first promoter is located upstream of ORF73, the coding region for the latent nuclear antigen (LANA) (Figure 1-4 – red arrows). The LANA promoter is constitutively active in many cell types, upregulated in S-phase of the cell-cycle and found to function primarily in B-cells in transgenic mice studies (Dittmer et al., 1998) (Sarid et al., 1999) (Jeong et al., 2002a).

Studies also demonstrate the potential for LANA to act on the LANA promoter indicating an auto-regulatory mechanism (Renne et al., 2001) (Jeong et al., 2004). The LANA promoter has also been shown to be transactivated by the lytic switch protein RTA, suggesting a role for RTA in establishing latency during de novo infection (Lan et al., 2005b). Transcription from the LANA promoter leads to the expression of ORF73 (LANA), ORF72 viral Fas-associated death domain (FADD) interleukin-1β-converting enzyme (FLICE) inhibitory protein (vFLIP) and ORF71 viral cyclin (v-cyc); via the differential splicing of polycistronic mRNAs and internal translation initiation sites (Figure 1-4) (Kedes et al., 1997b) (Grundhoff and Ganem, 2001) (Low et al., 2001) (Cesarman et al., 1996) (Bieleski and Talbot, 2001).

A second KSHV latent promoter transcribes an unspliced 1.3 kb mRNA which encodes the KSHV kaposin proteins (Figure 1-4 A - black arrow). The kaposins are translated from overlapping open reading frames that include the K12 sequence as well as two adjacent direct repeat elements, DR1 and DR2 (Figure 1-4 A) (Sadler et al., 1999). This mRNA also contains the viral miR-K10 miRNA. The remaining viral miRNAs are present within the intron of the latent kaposin pre-mRNA transcripts which initiate from a promoter located downstream of ORF72, as well as from the LANA promoter (Figure 1-4 A and B – white arrows and B – red arrows) (Li et al., 2002) (Cai and Cullen, 2006) (Pearce et al., 2005). The start site for the promoter denoted by the white arrow is more accurately defined by the position shown in Figure 1-4 B. Transcripts that terminate early from the promoter in the green circle potentially encode ORF71 and 72. The reason for having two promoters (red and white) that give rise to similar transcripts is not clear but may indicate cell-type differential regulation. The viral miRNAs are thought to be derived from the pre-mRNA by Drosha-mediated processing. Lastly, a transcript that originates from a novel promoter, LT_i, has been demonstrated as RTA responsive; again potentially indicating a role for RTA in establishing latency (Figure 1-4 – black arrow in blue circle) (Matsumura et al., 2005).

1.1.12.2 Latency-associated nuclear antigen (LANA)

KSHV ORF73 encodes the major latent protein LANA, translated from the tricistronic latency-associated transcript (LAT) (Kellam et al., 1997) (Kedes et

al., 1997b). LANA is a large (222-234 kDa) multi-functional protein known to interact with various cellular and viral proteins (Rainbow et al., 1997) (Verma et al., 2007). LANA has been detected in the nucleus of KSHV infected cells and in all forms of KSHV-associated malignancies (Dupin et al., 1999) (Gao et al., 1996a). The protein can be divided into three domains a C-terminal basic region involved in DNA-binding, a central repeat region of acidic amino acids and an N-terminal domain responsible for chromatin attachment and co-repressor recruitment (Lan et al., 2005a). A key function of LANA is the establishment and maintenance of the viral episome in the nucleus. LANA is involved in promoting semi-conservative replication of the circularised KSHV genome by binding to terminal repeat regions and recruiting host cellular DNA replication machinery (Garber et al., 2002) (Grundhoff and Ganem, 2003) (Lim et al., 2002). To ensure segregation of the viral genome into daughter cells during mitosis, LANA is also required to tether viral episomes to the host chromatin through binding to histones (Cotter and Robertson, 1999) (Ballestas et al., 1999) (Ballestas and Kaye, 2001) (Cotter et al., 2001) (Piolot et al., 2001) (Hu et al., 2002) (Ye et al., 2004) (Barbera et al., 2006). However, LANA is not sufficient for this process, this is demonstrated by the many failed attempts to culture KS spindle cell derived cell lines that maintain the episome (Flamand et al., 1996) (Dictor et al., 1996) (Grundhoff and Ganem, 2004). Therefore, stable latency is a multistage process that occurs with different efficiency in different cell types, requires cis-acting epigenetic changes and may rely on lytic replication to sustain a latently infected population of cells (Grundhoff and Ganem, 2004) (Pantry and Medveczky, 2009).

In addition to its role in KSHV plasmid replication and maintenance LANA has been implicated in the perturbation of several cellular pathways that regulate cell proliferation and survival. Expression of LANA under its own B-cell specific promoter in transgenic mice leads to splenic follicular hyperplasia and increased germinal centre formation (Fakhari et al., 2006). B-cell lymphomas, that did not resemble PEL, were seen at later stages highlighting the role of LANA in tumourigenesis. LANA physically binds to and represses p53-mediated transcription and apoptosis, promoting cell survival (Friborg et al., 1999). LANA also promotes cell cycle progression by binding to and inactivating the tumour suppressor retinoblastoma (Rb), increasing the expression of E2F dependent

genes. In combination with activate H-ras, LANA also led to the transformation of rat embryonic fibroblasts (Radkov et al., 2000). Via interactions with glycogen synthase kinase 3β (GSK-3β) LANA repositions the kinase to the nucleus where it can no longer degrade β-catenin in the cytoplasm (Krithivas et al., 2002) (Fujimuro and Hayward, 2003). The resulting accumulation of β-catenin can associate with lymphoid enhancing factor (LEF) and enter the nucleus to activate transcription of genes involved in cell cycle and oncogenesis, for example MYC. LANA was also shown to increase the expression of human telomerase reverse transcriptase (hTERT), prolonging the life span of primary cultured endothelial cells (Watanabe et al., 2003).

Many host and viral gene expression levels are manipulated by LANA expression (Renne et al., 2001). LANA primarily represses transcription by direct binding to DNA (Garber et al., 2002) and recruitment of the mSin3 co-repressor complex (Krithivas et al., 2000). Repression is achieved by binding and inhibiting the histone acetyl transferase (HAT) activity of the CREB binding protein (CBP) (Lim et al., 2001). LANA can directly inactivate transcriptional activators such as ATF4 (Lim et al., 2000). Finally, LANA has been shown to interact with the host recombination signal binding protein (RBP)-Jκ and target it to the promoter regions of lytic genes to mediate repression (Lan et al., 2005a). LANA therefore has important roles in viral genome maintenance, cell proliferation and survival as well as the repression of lytic transcripts, and is therefore critical for KSHV persistence.

1.1.12.3 v-Cyclin

Viral cyclin (v-cyclin) is the gene product of ORF72, translated from the abundant bicistronic LAT that also encodes ORF71. ORF72 is a viral homologue of cellular D-cyclin, which binds to and activates cyclin dependent kinase 6 (cdk6) as well as cdk4 to a lesser extent (Li et al., 1997). The v-cyclin/cdk6 complex is resistant to cdk inhibitors (cdki) and can phosphorylate a wider range of targets than its cellular counterpart including Rb, Histone H1 and the cdk inhibitor (cdki), p27 (Kip1), which is then targeted for degradation in cultured cells (Cesarman et al., 1996) (Chang et al., 1996) (Godden-Kent et al., 1997) (Ellis et al., 1999). This evidence indicates that v-cyclin is involved in promoting proliferation in KSHV infected cells. However, PEL cells have a high

level of the p27 and continue to proliferate (Carbone et al., 2000) (Jarviluoma et al., 2004) (Sarek et al., 2006) (reviewed in (Verschuren et al., 2004b)). In addition the cellular microRNAs miR-221 and miR-222 that are known to target p27, are absent in PEL indicating a more complex level of cell cycle regulation during latency (Visone et al., 2007) (Eve Coulter: personal communication).

Studies of v-cyclin are hampered by the inability to form stable cell lines overexpressing the protein (Ojala et al., 1999). Apoptosis is triggered in these cells due to v-cyclin/cdk6 inactivation of the anti-apoptotic factor, Bcl-2, adding a further layer of complexity to how v-cyclin can induce cell proliferation *in vivo* (Ojala et al., 2000). The oncogenic potential of v-cyclin has, however, been demonstrated in p53-/- transgenic mice that develop lymphomas, indicating the importance of LANA mediated inhibition of p53 in KSHV associated malignancy progression (Verschuren et al., 2002) (Verschuren et al., 2004a).

1.1.12.4 v-FLIP

ORF71 encodes the KSHV homologue of cellular Fas-associated death domain (FADD) interleukin-1β-converting enzyme (FLICE) inhibitory protein (FLIP). However, whether this protein functions to prevent FADD dependent caspase 8 recruitment to the death-inducing signalling complex (DISC), blocking apoptosis is unclear (Chaudhary et al., 2000). Several studies indicate that v-FLIP can directly interfere with the Fas-mediated apoptosis pathway, but this was not confirmed in later studies (Belanger et al., 2001) (Djerbi et al., 1999) (Chugh et al., 2005). A consistent observation, however, is the upregulation of the transcription factor, NF-κB (Chaudhary et al., 1999) (Matta et al., 2003). PEL cells have a constitutively active NF-κB pathway which has been attributed to the v-FLIP mediated activation of the IKK complex, leading to the phosphorylation of IκB and release of active NF-κB (Liu et al., 2002) (Field et al., 2003). This contributes to the proliferation of PEL cells, as knock down of v-FLIP in PEL cell lines or the inhibition of the proteasome blocking IκB degradation leads to cell death (Guasparri et al., 2004) (Matta and Chaudhary, 2005). NF-κB is also important in activation of the inflammatory response. This seems contradictory during latent infection, but may have relevance to KSHV-associated disease pathology (Li et al., 2005). Overexpression of v-FLIP in B-cells from transgenic mice led to increased responsiveness to mitogenic signal

as well as lymphoma development (Chugh et al., 2005). Forced expression in rodent fibroblasts also led to transformation, possibly indicating a role for v-FLIP interactions with tumour necrosis receptor-associated factor 2 (TRAF2) and receptor interacting protein (RIP) as well as activation of the JNK pathway (Sun et al., 2003) (An et al., 2003). v-FLIP inhibits lytic replication via inhibition of the AP-1 pathway indicating its role in the maintenance of latency in certain KSHV infected cell-types (Brown et al., 2003) (Ye et al., 2008) (Grossmann and Ganem, 2008).

1.1.12.5 Kaposins

The kaposin locus (K12 and surrounding direct repeat regions) encodes for at least 3 proteins via differential translation (Sadler et al., 1999). Kaposin A is a hydrophobic protein that localises to both surface and intracellular membranes (Tomkowicz et al., 2002). Overexpression in immortalised Rat-1 fibroblasts induced morphological transformation and highly vascular undifferentiated sarcomas, indicating a role in the deregulation of cell growth (Muralidhar et al., 1998). These changes are thought to be mediated via the interaction with cytohesin 1, a guanine exchange factor (GEF) for alternate reading frame (ARF) GTPases and a cell adhesion regulator (Kliche et al., 2001). Kaposin B is a small soluble protein localised to the nucleus, thought to act as a scaffold or adaptor protein. It causes the upregulation of various cytokines including IL6, tumour necrosis factor (TNF) and granulocyte-macrophage colony stimulating factor (GM-CSF) among others. This is achieved by the stabilisation of a class of cellular mRNAs containing AU-rich regions (i.e. cytokines); via the binding and activation of the mitogen-activated protein (MAP) kinase 2 (MK2) (McCormick and Ganem, 2005). Kaposin B represents another latent KSHV protein involved in inducing the inflammatory response. This, as discussed before, appears counterintuitive as viruses attempt to avoid the immune system, however; this response may be required to maintain latency *in vivo* or used to recruit susceptible cells to the site of infection, facilitating spread of the virus. Kaposin C shares a common repeat amino acid sequence with kaposin B and is therefore a trans-membrane fusion protein.

1.1.12.6 LANA2/vIRF3

The KSHV genome encodes four viral homologues of cellular interferon regulatory proteins (vIRFs) which cluster in one region; suggestive of ancestral gene duplication probably after acquisition from the host. The IRFs are a family of transcription factors involved in regulating the interferon response (Barnes et al., 2002). Viral IRFs therefore contribute to immune-evasion and are lytic proteins except for K10.5 or LANA2 (vIRF3); which is expressed predominantly during latency, but can be induced during lytic reactivation (Lubyova and Pitha, 2000). LANA2 is expressed exclusively in PEL and MCD, indicating the potential of different KSHV latency programs (Rivas et al., 2001), and has been shown to be essential for PEL cell survival (Wies et al., 2008). This nuclear protein has a role in the disruption of cellular IRF-7, IRF-3 and IRF-5 mediated signalling and cellular PKR signalling (Esteban et al., 2003) (Joo et al., 2007) (Wies et al., 2009). However, like some cellular IRFs for example IRF4, LANA2 also has a role in cell growth and regulation and has been shown to inhibit p53 and NF-κB as well as stimulate MYC expression. This highlights the importance of LANA2 in KSHV pathogenesis and cell proliferation (Rivas et al., 2001) (Seo et al., 2004) (Lubyova et al., 2007).

1.1.12.7 Viral miRNA

MicroRNAs (miRNA) are a family of recently discovered non-coding RNAs, which are approximately 21-22 nucleotides in length and play an important part in post-transcriptional gene regulation (reviewed in (Bartel, 2004)). This is a highly conserved mechanism of regulating gene expression and several herpesviruses have been shown to also express viral miRNAs (Pfeffer et al., 2005) (Swaminathan, 2008) (Umbach and Cullen, 2009). During KSHV latency, 18 mature miRNAs are processed from 12 pre-miRNAs, 14 of which are present in the same genome region, or cluster, and are expressed from a single precursor mRNA (see section 1.1.12.1) (Cai et al., 2005) (Pfeffer et al., 2005) (Samols et al., 2005) (Grundhoff et al., 2006) (Umbach and Cullen, 2010). The cellular targets of these viral miRNAs can be predicted by looking for binding sequences in the 3'UTR of cellular mRNAs. However, few potential targets have been experimentally confirmed as yet (reviewed in (Bartel, 2009)). Despite this, viral miRNAs are predominantly involved with regulating lytic reactivation, apoptosis, immune evasion, cellular differentiation and reprogramming

(reviewed in (Umbach and Cullen, 2009)) (Hansen et al., 2010). In one instance, KSHV-miR-K12-11 has been shown to function as an orthologue (shares 100 % seed sequence homology) of cellular miR-155, which is in fact absent from PEL cells (Gottwein et al., 2007) (Skalsky et al., 2007). This suggests a viral miRNA is able to target the same mRNAs as cellular miR-155, including the transcriptional repressor BACH-1, and regulate the same processes such as germinal centre development and the generation of antigen-specific switched antibody-producing cells (Calame, 2007). However, overexpression of miR-155 triggers the development of B cell malignancies, and is abundantly expressed in diffuse large B-cell lymphomas (DLBCLs) (Kluiver et al., 2005).

Most recently, a specific role for KSHV miRNAs in the regulation of the switch between latent and lytic replication phases has been established. KSHV miR-K9* has been shown to target the 3'UTR of the immediate early viral RTA protein, necessary and sufficient for lytic reactivation (Bellare and Ganem, 2009) (Lukac et al., 1998) (Xu et al., 2005). The ectopic expression of miR-K9* reduced the expression of a luciferase reporter driven by the RTA 3'UTR, and expression of an antagonist to miR-K9* in a KSHV latent cell system led to increased lytic replication (Bellare and Ganem, 2009). This viral miRNA therefore acts to maintain latency. Previous studies of miR-K5 and its cellular target Bcl-2-associated transcription factor 1, BCLAF-1, showed an increase in sensitivity to lytic reactivation (Ziegelbauer et al., 2009). Additionally, another KSHV miRNA, miR-K1 has been shown to target IκBα, an inhibitor of the NF-κB pathway, which leads to increased NF-κB activity and increased lytic replication (Lei et al., 2010). Finally, KSHV miR-K12-5 maintains latency by reducing levels of mRNA encoding RTA, while the KSHV miR-K12-4-5-p was found to target a Retinoblastoma-like protein 2 (Rbl2) a repressor of the DNA methyltransferases (DNMT) 3a and b, making global epigenetic changes (Lu et al., 2010). The various examples of viral miRNA regulation of lytic replication, outlined above, suggest that they all act to 'fine tune' the level of reactivation, a vital and already highly controlled process (see section 1.1.14), allowing the virus to intricately respond to the changing cellular environment. These results demonstrate that the gene expression profile of a cell infected with an oncogenic miRNA expressing virus, like KSHV, is dependent on both the virus and host miRNA

expression, introducing yet another layer of virus-host interaction (Umbach and Cullen, 2010).

1.1.13 KSHV Lytic cycle

Within the predominantly latent population of KS spindle cells around 3 % of cells express markers of lytic replication (Parravicini et al., 2000) (Katano et al., 2000). This small proportion of cells is thought to contribute to the pathogenesis of KS in two main ways. Firstly, the retention of KSHV genomes in dividing cells, other than B-cells, is inefficient and therefore re-infection of endothelial cells is required for viral persistence (Grundhoff and Ganem, 2004). Secondly, many of the genes expressed during the lytic cycle induce the expression of host cellular cytokines which are responsible for the angiogenesis and cell proliferation seen in KS. The chemokine receptor homologue vGPCR which induces the expression of VEGF is an example of this (Arvanitakis et al., 1997) (Montaner et al., 2003).

Table 1-2 Classification of KSHV lytic genes

Class	Symbol	Definition
Immediate-early (IE)	α	Expression requires no new viral protein synthesis (transcription insensitive to cycloheximide).
Delayed-early (DE)	β	Expression is independent of virus DNA synthesis (transcription prevented by cycloheximide but insensitive to DNA synthesis inhibitors). Depend on IE gene function.
Partial-late (L)	$\gamma 1$	Expression is increased by viral DNA synthesis (transcription prevented by cycloheximide and reduced by DNA synthesis inhibitors.
True-late (L)	$\gamma 2$	Expression requires viral DNA synthesis (transcription blocked by cycloheximide or DNA synthesis inhibitors).

Lytic replication results in the production of new viruses and therefore involves viral DNA replication by the virus-encoded replication machinery, packaging of virus genomes into newly assembled capsids and subsequent maturation into a viral particle (see sections1.1.13.1 and 1.1.5). This occurs via a highly regulated and temporally ordered gene expression cascade that is initiated by the viral lytic switch protein, RTA (see section 1.1.14). Genes expressed during the lytic cycle can be grouped based on their timing and their expression in response to protein synthesis and DNA replication inhibitors (Zhu et al., 1999) (Saveliev et

al., 2002) (Lu et al., 2004a) (see Table 1-2). The immediate early (IE) genes are regulatory and include ORF50 encoding RTA, which transactivates the promoters of the delayed early genes (DE). Many DE genes have enzymatic functions, for example DNA polymerase, or are accessory proteins, such as the polymerase processivity factor. DE genes are primarily involved in viral DNA replication and protein production. Functions of KSHV DE genes also include regulation of gene expression for example, the mRNA transcript accumulation (MTA) protein (Kirshner et al., 2000). Immune evasion is another important function of DE genes (reviewed in (Coscoy, 2007)) for example: the modulators of immune recognition (MIR1/2) and the membrane bound E3-ubiquitin ligases (encoded by ORFK3 and -K5), which evade CTL recognition by removing MHC class I from the surface of infected cells via endocytosis and subsequent endosmomal degradation (Coscoy and Ganem, 2000) (Coscoy et al., 2001). K5 has also been shown to downregulate BST2/tetherin from the cell surface; facilitating virus release from cell membranes (Mansouri et al., 2009) (Pardieu et al., 2010). KSHV also encodes several viral homologues of interferon regulatory factors (vIRFs) which are capable of opposing the function of cellular IRFs and suppress interferon responses (reviewed in (Lee et al., 2009)). Remaining functions of DE genes include the anti-apoptotic role of the viral homolog of Bcl-2 (v-Bcl-2) (Sarid et al., 1997), selective accelerated turnover of host mRNA mediated by ORF37, shutoff exonuclease (SOX) (Glaunsinger et al., 2005), and the modulation of signal transduction carried out by various virally encoded proteins including K1. K1 encodes a type I transmembrane protein with an immunoreceptor tyrosine-based activation motif (ITAM) and mimics a constitutively active B-cell antigen receptor (Lee et al., 1998) (Lagunoff et al., 1999). ORFK2 encodes a viral homologue of cellular interleukin 6 (vIL-6), this protein triggers JAK/STAT signalling leading to the upregulation of VEGF (Molden et al., 1997). Other virally encoded chemokines, the macrophage inflammatory proteins (vMIPs), are involved in polarisation of the immune response away from the Th1 driven anti-viral response (Choi and Nicholas, 2008). Another KSHV protein known to induce VEGF is the constitutively active homologue of the cellular IL-8 receptor, G-protein coupled receptor (ORF74/vGPCR), which has a broad range of signalling activity leading to proliferation and angiogenesis (Cannon et al., 2003). Finally, the late genes (L) are transcribed following DNA replication and provide the structural genes

for virus assembly for example, the major capsid protein (MCP) (Chang and Ganem, 2000) (Jenner et al., 2001).

Cell cycle arrest at the G1/S boundary is associated with lytic infection by herpesviruses, including KSHV, where the replication associated protein (RAP) or K-bZIP/K8 is mechanistically involved (Wu et al., 2002) (Izumiya et al., 2003b). K-bZIP increases the accumulation of C/EBP-α leading to cell cycle arrest via the upregulation of the cdk inhibitor p21 and the inhibition of E2F expression (Wu et al., 2003). K-bZIP is also able to directly bind to cdk2/cyclinA/E complexes, which causes impaired kinase activity (Izumiya et al., 2003b). This block to the cell cycle prevents cellular DNA synthesis, reducing competition for nucleotide pools and increasing the access to sub-nuclear compartments required for replication.

1.1.13.1 KSHV lytic DNA replication

Information on KSHV lytic DNA replication is derived from studies of other herpesviruses including EBV and HSV, whose DNA replication had been reconstructed *in vitro* (Boehmer and Lehman, 1997). DNA replication involves a multi-subunit complex composed of the catalytic and accessory proteins required for DNA synthesis from a cis-acting replication origin. KSHV encodes homologues of the six components of the core machinery of viral DNA replication, viral DNA polymerase (ORF9), primase (ORF56), polymerase processivity factor (ORF59), primase-associated factor (ORF40/41), helicase (ORF44) and single stranded binding protein (ORF6) in abundance. These proteins when co-expressed in the absence of viral genome form a stable complex in the nucleus (Wu et al., 2001a). KSHV-encoded K8 (ORFK8/K-bZIP) protein, which is a distant evolutionary homologue to EBV ZTA, was also incorporated into these structures. Once genomic template is present, DNA replication will occur via a rolling circle mechanism in a replication compartment usually found in the vicinity of a host ND10 body, a site of viral DNA accumulation (Ishov and Maul, 1996). The multimeric genomes produced require processing into linear monomers before packaging into nascent capsids.

KSHV has two origins of lytic replication (Ori-Lyt) which have close sequence homology. The left hand origin (Ori-Lyt-L) is located between ORFK4.2 and -K5

and the right hand origin (Ori-Lyt-R) is found between ORFK12 and ORF71 (AuCoin et al., 2002) (see Figure 1-1). Ori-Lyt-L is sufficient for viral genome propagation whereas Ori-Lyt-R, on its own, is unable to direct amplification of viral DNA (Xu et al., 2006). For an origin of replication to be functional, four critical elements are required, four pairs of C/EBPα binding sites, an 18 bp AT-rich palindrome, a 32 bp sequence of as yet unknown function and a high affinity binding site for RTA near a TATA box (AuCoin et al., 2004) (Wang et al., 2004c). RTA and K-bZIP are required for DNA replication from the Ori-Lyt, and RBP-Jκ is needed to overcome LANA mediated repression of DNA replication (AuCoin et al., 2004) (Wang et al., 2004c) (Wang et al., 2006) (Lefort and Flamand, 2009) (Rossetto et al., 2009) (see section 1.1.14.). Herpesviruses also encode proteins involved in DNA repair and nucleotide metabolism which are essential for *in vivo* replication (Coleman et al., 2003). Various cellular proteins are involved in KSHV DNA replication including topoisomerase (Topo) I and II, which are essential (Wang et al., 2008).

1.1.14 Reactivation from latency

An important aspect of the definition of latency is the ability to reactivate and produce progeny virions. This is required for viral persistence, transmission and disease pathogenesis and therefore is studied intensely for the discovery of therapeutic targets. The process of switching from latent, non-productive infection to lytic replication is known as reactivation. In KSHV this is governed by ORF50 that encodes the immediate early regulator of transcription activation, RTA (Zhu et al., 1999). Ectopic expression of RTA is sufficient to induce reactivation in latently infected cells, and removal of RTA or inhibition of its function prevents spontaneous and chemically induced reactivation (Gradoville et al., 2000) (Lukac et al., 1998) (Sun et al., 1998) (Lukac et al., 1999) (Xu et al., 2005). RTA in combination with other viral and cellular transcription factors (see Table 1-3) (Wang et al., 2001c) is able to transactivate its own promoter and those of many viral and cellular genes, initiating a highly regulated gene expression cascade that results in the manufacture of new virions (see section 1.1.13) (Deng et al., 2000) (reviewed in (West and Wood, 2003)). RTA is known as a KSHV 'molecular switch' that controls reactivation. Factors affecting the expression of this protein therefore impact on the balance between KSHV lytic and latent replication (see Table 1-3 and Table 1-4).

RTA is transcribed from the major 3.6 kb transcript of the ORF50 region. Minor, smaller RNA species are also transcribed from this region and are complementary in sequence to RTA mRNA. They are expressed during reactivation but do not appear to encode any viral proteins, their role in lytic replication is therefore unclear (Lukac et al., 1999) (Zhu et al., 1999) (Saveliev et al., 2002). The RTA protein is organised in the same manner as other general viral transcriptional activators. Similar to its functional homolog in EBV, RTA has an N-terminal DNA-binding domain and a potent transcriptional activator at the C-terminus (Manet et al., 1991) (Hardwick et al., 1992) (Lukac et al., 1999). Deletion of the C-terminal amino acids 531-691 removes the transactivation domain and creates a dominant negative (dn) mutant RTA (Lukac et al., 1999) (discussed further in Chapter 5). An N-terminal proline-rich, leucine heptapeptide repeat (LR) region is important for RTA multimerisation and RTA is thought to function most efficiently as a tetramer (Bu et al., 2007).

RTA can transactivate either by direct binding to RTA response elements (RRE), which have limited consensus, or via cooperative interactions with partners such as RBP-Jκ (Song et al., 2001) (Liao et al., 2003a) (Ziegelbauer et al., 2006) (Persson and Wilson, 2010). RTA is also highly phosphorylated but the function of this post-translational modification has not yet been identified (Lukac et al., 1999).

Numerous factors control the expression of RTA and therefore KSHV lytic replication. These include both epigenetic modifications (Pantry and Medveczky, 2009) as well as cellular signalling pathways and these are outlined in Table 1-3 and Table 1-4 below. Over 20 stimuli are reported to disrupt RTA regulation and cause KSHV reactivation; however the physiological relevance of many of these stimuli is yet to be explained (see Table 1-3). Many are also involved in RTA transcriptional regulation of other viral and cellular proteins and are therefore important for lytic replication.

Table 1-3 Factors reported to induce RTA expression and RTA dependent lytic gene expression

Factor	Mechanism	Reference
12-O-tetradecanoyl phorbol-13-acetate (TPA)	Requires activation of protein kinase Cδ (Deutsch et al., 2004) and requires activating protein 1 (AP-1) (Wang et al., 2004b). Role for MAPK pathways.	(Renne et al., 1996b) (Moore et al., 1996a) (Cohen et al., 2006)
Sodium butyrate	The ORF50 promoter is present in a region of densely packed chromatin. Sodium butyrate inhibits histone deacetylases causing chromatin remodelling of ORF50 promoter via recruitment of Ini1/Snf5 (Lu et al., 2003) and recruitment of Sp1 and Sp3 complexes (Ye et al., 2005)	(Miller et al., 1997) (Miller et al., 1996)
Ionomycin	Mobilises Ca^{2+} and reactivation is dependent on calcineurin (Zoeteweij et al., 2001)	(Chang et al., 2000)
Thapsigargin	Mobilises Ca^{2+} and reactivation is dependent on calcineurin (Zoeteweij et al., 2001)	(Zoeteweij et al., 2001)
5-azacytidine	The ORF50 promoter is heavy methylated in a asymptomatic individuals (Chen et al., 2001). Demethylating agents therefore modify the chromatin facilitating reactivation	(Chen et al., 2001)
Prostratin	A non-tumour-promoting phorbol ester	(Brown et al., 2005)
Bortezomib	Proteasome inhibitor	(Brown et al., 2005)
Valproic acid	Inhibits histone deacetylases	(Shaw et al., 2000)
Trichostatin A	Inhibits histone deacetylases	(Lu et al., 2003)
Hydrocortisone	Direct activation of KSHV lytic gene transcription	(Hudnall et al., 1999)
Hypoxia	Hypoxia inducible factors (HIFs) activate the ORF50 promoter (Haque et al., 2003)	(Davis et al., 2001) (Cai et al., 2006a)
Epithelial differentiation		(Johnson et al., 2005a)

Plasma cell differentiation	XBP-1 transactivates the ORF50 promoter	(Wilson et al., 2007) (Sun and Thorley-Lawson, 2007) (Yu et al., 2007b) (Liang et al., 2009) (Siegel et al., 2010)
Catecholamine neurotransmitters	Physiological levels of epinephrine and norepinephrine act via B adrenergic activation of protein kinase A (PKA)	(Chang et al., 2005)
Dopamine receptor targets	Via activation of protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) signalling pathways	(Lee et al., 2008)
MAPK	Ras/Raf/MEK/ERK, JNK and p38: Ets-1 and AP-1 mediated transactivation of KSHV ORF50 (Pan et al., 2006)	(Yu et al., 2007b) (Xie et al., 2008)
Inflammatory cytokines	Including Interferon-γ, Oncostatin M, Hepatocyte growth factor/ scatter factor and Interleukin-6, possibly via the action of the cellular Kinase Pim-1 (Cheng et al., 2009b)	(Blackbourn et al., 2000a) (Chang et al., 2000) (Mercader et al., 2000) (Monini et al., 1999)
Pim-1/Pim3 kinases	Phosphorylation of LANA counteracts its inhibition of transcription from the TR region	(Cheng et al., 2009b)
TLR signalling	Activation of toll like receptor (TLR) 7/8	(Gregory et al., 2009)
HIV-1 tat	Via JAK/STAT signalling (Zeng et al., 2007)	(Harrington et al., 1997)
KSHV RTA	Auto activates RTA expression (Deng et al., 2000)	(Lukac et al., 1998)
K-bZIP/K8	Knock down K-bZIP see loss of ORF 50 transcript in PEL cells	(Lefort and Flamand, 2009)
ORF57/MTA	Ability to transactivate promoters, either alone or synergistically with RTA, is cell and promoter specific. Does not induce reactivation alone	(Malik et al., 2004) (Palmeri et al., 2007)

RTA homologues	HVS, MHV68, RRV via direct transactivation of ORF50	(Goodwin et al., 2001) (Damania et al., 2004)
Co-infection	HHV6, HSV-1, HIV-1 and HCMV in human fibroblasts but not in PEL	(Lu et al., 2005) (Qin et al., 2008) (Merat et al., 2002) (Vieira et al., 2001) (Cheng et al., 2009a)
cJun	Via direct transactivation of the ORF50 promoter	(Gwack et al., 2001)
cFOS-cJUN heterodimer/ activating protein 1 (AP-1)	Via direct transactivation of the ORF50 promoter and cooperates with RTA	(Wang et al., 2004b)
CCAAT/ Enhancer-binding protein-α (C/EBPα)	Exogenously expressed C/EBPα can induce RTA expression and cooperates with RTA	(Wang et al., 2003b) (Wang et al., 2003a)
Recombination signal-binding protein 1 for J-kappa (RBP-Jκ)/CSL	RTA interacts with the primary target of the Notch signalling pathway to direct RTA to target promoters. The strong transactivator domain of RTA overrides the repressive function RBP-Jκ by displacing repressive complexes and recruiting coactivator complexes	(Liang et al., 2002) (Persson and Wilson, 2010)
Intracellular domain of Notch1 (ICN)	Ectopic expression induces KSHV lytic replication and requires RBP-Jκ binding sites in the ORF50 promoter	(Lan et al., 2006)
MGC2663/ KSHV-RTA binding protein (K-RBP)	Synergises with RTA weakly enhancing RTA-mediated transactivation	(Wang et al., 2001c)
Octamer-binding protein 1 (Oct-1)	Binds octamer sequences in the ORF50 promoter and enhances RTA-mediated transactivation. Also cooperates with RTA.	(Sakakibara et al., 2001) (Carroll et al., 2007)

cAMP response element (CRE)-binding protein (CBP)	Via direct transactivation of the ORF50 promoter and via histone acetyl transferase (HAT) activity transforms the chromatin into a more accessible state	(Lu et al., 2003) (Gwack et al., 2001)

In addition to the factors that induce directly or cooperate with RTA to facilitate reactivation many factors have been identified that repress RTA functions (see Table 1-4). Many of these factors are therefore important for the maintenance of latency. For example, LANA which interacts with and inhibits many of the factors designated as inducers or co-operators of RTA.

Table 1-4 Factors reported to negatively regulate RTA

Factor	**Mechanism**	**Reference**
RBP-Jκ/CSL	Interacts with LANA to mediate ORF50 repression	(Lan et al., 2005a)
K-bZIP/K8	Interacts with RTA and represses RTA-mediated transactivation of K8 promoter. Interacts with CBP repressing AP-1 mediated transactivation	(Liao et al., 2003b) (Hwang et al., 2001) (Izumiya et al., 2003a)
MGC2663/ KSHV-RTA binding protein (K-RBP)	K-RBP was found to repress RTA-mediated transactivation, however RTA can promote K-RBP degradation	(Yang and Wood, 2007) (Yang et al., 2008)
LANA	Interferes with RTA mediated promoter activation through interactions with, RTA, RBP-Jκ, ICN and CBP	(Lan et al., 2004) (Lan et al., 2005a) (Lan et al., 2006) (Lim et al., 2001)
Histone deacetylase -1 (HDAC1)	Interacts with the ORF50 promoter and mediates repression	(Gwack et al., 2001) (Lu et al., 2003)
Nuclear factor kappa B (NF-κB)	Inhibits RTA mediated transactivation in a context dependent manner	(Brown et al., 2003) (Grossmann and Ganem, 2008) (Izumiya et al., 2009) (Lei et al., 2010)

Interferon regulatory factor 7 (IRF7)	Competes with RTA for binding to the RTA response element in the ORF57 promoter	(Wang et al., 2005a)
Hairy/E(spl)-related with YRPW (Hey1)	Induced by RTA, Hey1 represses the ORF50 promoter	(Yada et al., 2006)
Poly(ADP-ribose) polymerase 1 (PARP-1)	Efficiently transfer of poly(ADP-ribose) to RTA causing repression	(Gwack et al., 2003)
Ste20-like kinase (hKFC)	Efficiently transfer of phosphate units to RTA causing repression	(Gwack et al., 2003)
Phosphatidylinositol 3-kinase (PI3K)-Akt	Suppresses the activity of RTA. Inhibitors of the pathway enhances response to TPA	(Peng et al., 2010)
Octamer-binding protein 2 (Oct-2)	Competes with Oct-1 for the octamer binding motif present in the ORF50 promoter	(Di Bartolo et al., 2009)
KSHV microRNAs	Direct targeting of 3' UTR of RTA and increases methyltransferase activity. Provides 'fine-tuning' regulation of RTA expression	(Bellare and Ganem, 2009) (Lu et al., 2010)

KSHV reactivation is a complex and highly regulated process. The balance between latency and lytic replication is therefore highly sensitive and can be seen as equilibrium between the induction and repression of lytic gene expression, controlled by the expression of RTA. The ability of RTA to respond to a multitude of different stimuli *in vitro* indicates the capacity of KSHV to reactivate in response to many different scenarios *in vivo*. Alternatively, the effect of many negative regulators suggests that the KSHV lytic cycle is robustly buffered against inappropriate lytic reactivation. This implies that the most important pathways, that induce reactivation from latency, are those that are physiologically relevant to KSHV infection and the associated diseases. These, triggers of lytic replication, represent potential targets for therapy and disease intervention.

1.1.14.1 A comparison with EBV reactivation from Latency

In contrast to KSHV, where RTA is both necessary and sufficient for lytic reactivation, activation of the EBV lytic cycle is mediated by two viral proteins proteins. However, analogously to RTA the expression of immediate early viral protein BZLF-1 (ZTA) is a primary event sufficient to trigger the entire EBV lytic cascade (Countryman and Miller, 1985). BZLF-1 is a bZIP transcription factor related to KSHV ORFK8/K-bZIP but is a functional homology of KSHV RTA (Sinclair, 2003) (Chang et al., 1990). Like RTA, BZLF-1 is able to autoactivate its own promoter as well as a subset of EBV genes including the second viral protein important for EBV reactivation, BRLF-1 (RTA), through ZTA response elements (ZREs) (Hardwick et al., 1988) (Speck et al., 1997). BZLF-1 transactivation of BRLF-1 is thought to be enhanced by promoter methylation, a feature that KSHV RTA is as yet not known to do (Bhende et al., 2004). Phosphorylation of BZLF-1 has also been shown to enhance transactivation capabilities, a trait thought to be important for RTA (Kolman et al., 1993) (Chang et al., 2005). The expression of BRLF-1 requires protein synthesis and therefore can not be classified as immediate early (Amon et al., 2004). However, BRLF-1 plays an important cooperative role alongside BZLF-1 in transactivation of viral genes in the next stage of lytic replication (Quinlivan et al., 1993). During latency the BZLF-1 promoter (Zp) is inactivated by repressive chromatin structure that can be released by histone acetylation for example as a result of BCR signalling, a mechanism of reactivation that is also used in KSHV reactivation (Jenkins et al., 2000) (Bryant and Farrell, 2002).

1.2 Cellular stress

Hans Selye first described physiological stress and suggested a ‘common response of the body to a variety of insults and “stressful” conditions’. Cellular stress has since been defined as a ‘variety of processes that are triggered by an acute or chronic shift from the usual cellular conditions and homeostasis that aim to counteract the insult, repair the damage and eventually protect the cell or organism’ (Barouki and Sitia, 2007). This definition indicates that there are many conditions that alter the cellular environment and activate a variety of responses required for cell adaptation and recovery.

Cellular stress may be caused by exogenous factors such as extremes of temperature, radiation, hypoxia, nutrient deprivation, free radicals, toxins/chemicals and pathogen infection. There are also endogenous sources of cell stress, for example the disruption of protein folding in the endoplasmic reticulum (ER) during plasma cell differentiation, which leads to ER stress termed 'physiological stress' (Rutkowski and Kaufman, 2004). In some instances the same cellular stress response can be induced by both exogenous and endogenous factors to different levels of effect and control. However, the cell is able to distinguish between these situations and respond accordingly with highly regulated response programmes. For example, the unfolded protein response (UPR) is the cellular stress response to ER stress (see section 1.3). This adaptive response may be elicited by a temporary environmental insult, for example hypoxia, which requires activation of the UPR to allow cells to recover or remove cells with irrevocable damage. However, chronic ER stress occurs in professional secretory cells, such as plasma cells, hepatocytes and β-cells of the pancreas. Here the UPR is physiologically activated to facilitate development and survival of these cell types that have a high secretory capacity. Therefore, classical regulation of the UPR (described in section 1.3) provides general cellular protection to acute ER stress. However, each pathway has diverse roles within the response and can be selectively activated in different tissues to provide a tailored response to various physiological and pathological induced stresses (reviewed in (Wu and Kaufman, 2006)). The physiological role of the UPR in development and function is best understood in the context of professional secretory cells and in cancer progression (see sections 1.4 and 1.5). Virus infection not only contributes to cellular stress but due to their dependence on the host cell, viruses are consequently also susceptible to the many cellular stresses encountered. How the virus life cycle reacts to the various changes that occur within the host cell, in response to cellular stress, highlights an important aspect of virus-host interaction. This aspect is investigated by this thesis, in the context of physiological stresses that can trigger reactivation of latent virus from infected cells.

1.3 The unfolded protein response

The unfolded protein response (UPR) is an evolutionarily conserved integrated signal transduction pathway that mediates the adaptation to protein-folding

stress (reviewed in (Ron and Walter, 2007)). The endoplasmic reticulum (ER) receives newly synthesised polypeptide chains destined for secretion or the cell-surface, and provides an optimum environment for the correct folding and assembly, allowing these proteins to mature. However, the nascent protein load of the ER varies, sometimes rapidly, in response to an array of insults including exposure to pharmacological agents that disturb protein folding, genetic mutation of ER chaperones or chaperone substrates, failure of post-translational modifications, alterations to the reduction-oxidation (redox) balance of the ER, changes in calcium concentration, pathogen infection, nutrient deprivation, as well as normal differentiation and function of professional secretory cells. ER stress occurs when the amount of unfolded protein in the ER is greater than the capacity of the cellular machinery to deal with this protein load. The cellular response is to restore homeostasis by increasing the protein-folding capacity of the ER and to trigger apoptosis when the system remains overwhelmed. This is coordinated by the UPR which: (i) reduces the amount of newly synthesised protein delivered to the ER; (ii) upregulates the expression of chaperones and foldases as well as proteins involved in ER entry and degradation pathways; (iii) induces the expansion of the secretory apparatus involving lipid biogenesis and increases vesicle trafficking; (iv) regulates cell cycle progression, autophagy and apoptosis (reviewed in (Schroder and Kaufman, 2005)). Many diseases are associated with chronic ER stress including neurodegenerative disorders (reviewed in (Matus et al., 2008)), cancer (reviewed in (Moenner et al., 2007)), inflammation and autoimmunity (reviewed in (Todd et al., 2008)) (Yoshida, 2007) and diabetes (reviewed in (Lipson et al., 2006)). Recent studies have linked insulin signalling via the PI3K pathway directly to the UPR via the interaction of a PI3K regulatory subunit and X-box binding protein-1 (XBP-1); see section 1.3.3.1 (Winnay et al., 2010). This interaction is required for the nuclear translocation of XBP-1 and adaptation to metabolic induced ER-stress, thereby preventing the development of insulin resistance (Park et al., 2010). Therefore, uncovering the mechanisms of UPR regulation in these disease states allows further insight into the response and may uncover novel therapeutic strategies.

The UPR was originally discovered in budding yeast cells as a single pathway mediated by inositol-requiring kinase/endonuclease-1 (IRE-1), a type 1 ER-

resident transmembrane protein (reviewed in (Patil and Walter, 2001)). Higher-order eukaryotic organisms have evolved a more complex UPR with three separate branches. Signalling is initiated by three distinct ER integral membrane proteins, activating transcription factor-6 (ATF6), protein kinase RNA (PKR)-like ER kinase (PERK) and the conserved IRE-1α (see Figure 1-5). The UPR in mammalian systems is however highly coordinated and there is a degree of cross-talk between the pathways which includes redundancy, positive reinforcement and negative feedback (reviewed in (Ron and Walter, 2007)).

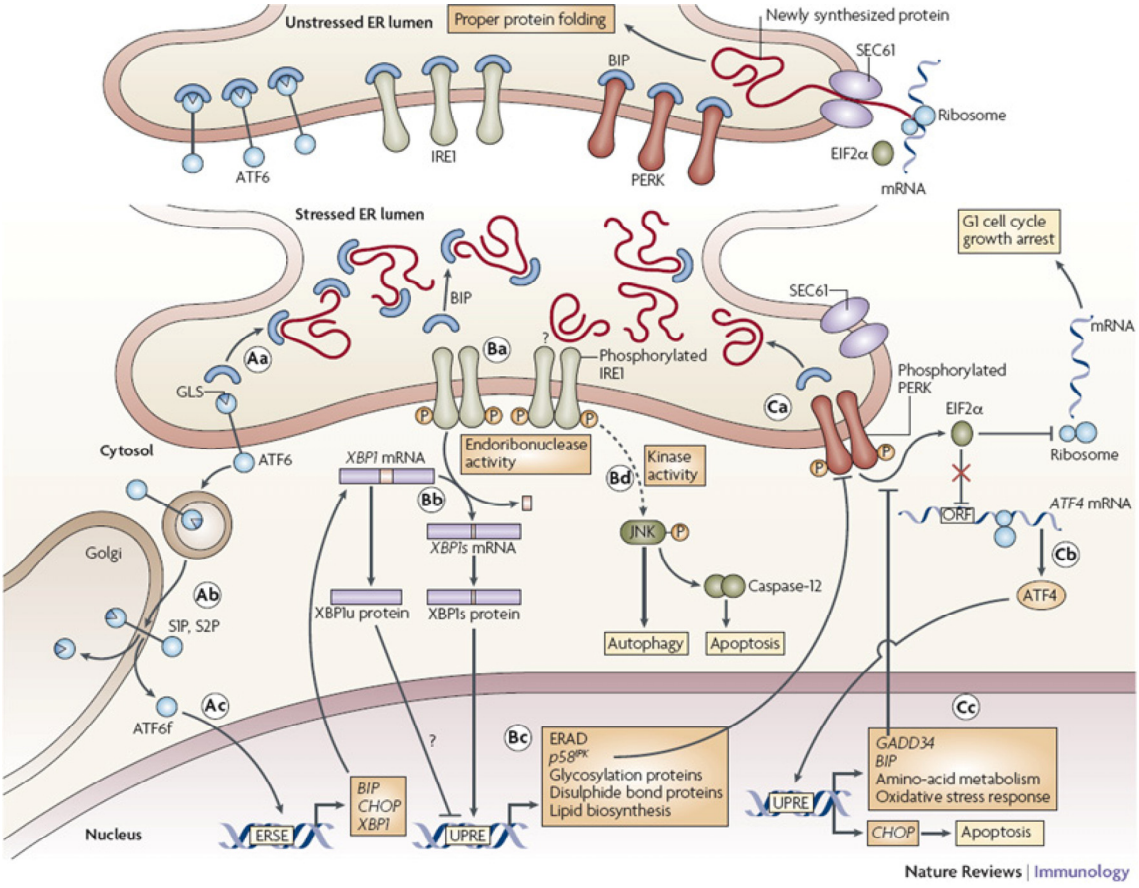

Figure 1-5 The mammalian unfolded protein response
During unstressed conditions (top panel), BiP binds to and inhibits the three endoplasmic reticulum (ER)-proximal unfolded-protein response (UPR) transmembrane proteins: activating transcription factor 6 (ATF6), inositol-requiring transmembrane kinase/endonuclease 1 (IRE1) and pancreatic ER kinase (PERK). ER stress is averted as long as newly synthesized proteins fold properly as they transit into the ER lumen through SEC61 channel proteins. When unfolded proteins accumulate during stress (bottom panel), BiP is recruited away from ATF6, IRE1 and PERK. This has the potential to activate the different UPR signalling axes. A | The release of BiP exposes a Golgi-localization sequence (GLS) within ATF6 (Aa), targeting the molecule for the Golgi. In the Golgi, ATF6 is sequentially cleaved by site-1 protease (S1P) and S2P (Ab). This releases the ATF6 fragment (ATF6f) transcription factor, which translocates to the nucleus, binds to ER-stress response elements (ERSE) and induces transcription of several genes, including BIP, CHOP (CCAAT/enhancer-binding protein, homologous protein) and X-box binding protein 1 (XBP-1) (Ac). (B) The release of BiP from IRE1 allows for homodimerisation and activation of IRE1 through autophosphorylation. Unfolded proteins themselves may also directly activate IRE1 (Ba). Phosphorylated IRE1 possesses endoribonuclease activity that excises a 26 base pair fragment from unspliced XBP-1 mRNA and, following religation by a putative tRNA ligase, forms spliced XBP-1s mRNA (Bb). XBP-1s protein translocates to the nucleus, where it binds to UPR elements (UPREs) and activates many genes that are crucial for

secretory function. One of these gene products, $p58^{IPK}$, inhibits PERK activity. Although XBP-1u protein is short-lived, it can impair XBP1s transcriptional function (Bc). In addition to its endoribonuclease activity, phosphorylated IRE1 also leads to activation of JUN N-terminal kinase (JNK), through TRAF2 (tumour-necrosis factor (TNF)-receptor-associated factor 2) and ASK1 (apoptosis signal-regulating kinase 1; not shown), which can promote cell survival by inducing autophagy or can lead to programmed cell death through caspase-12-mediated apoptosis pathways (Bd). C | Much like IRE1, PERK is activated by autophosphorylation following the release of BiP. Phosphorylated PERK also phosphorylates eukaryotic translation-initiation factor-2α (eIF-2α), causing translational arrest of most proteins and subsequent cell cycle growth arrest (Ca). However, ATF4 mRNA escapes translational suppression because it possesses upstream open reading frames (ORFs) (Cb). ATF4 translocates to the nucleus, where it activates a third set of UPR target genes (Cc). These include GADD34 (growth-arrest DNA damage gene 34), which feedbacks to inhibit PERK, and CHOP, which can induce apoptosis in cells with irrecoverable levels of ER stress. Although each UPR pathway induces different target genes, in total they allow a cell to combat the endogenous stress of unfolded proteins by shutting off the synthesis of new proteins. They also enable a cell to export existing proteins by increasing the secretory capacity of the cell, ERAD, ER-associated degradation. Taken from (Todd et al., 2008).

In the absence of stress, the ER-lumenal domains of these signal transducers are bound by the IgH binding protein, BiP/GRP78, and held in an inactive state (Bertolotti et al., 2000) (see Figure 1-5 top panel). However BiP, an ER chaperone, has an affinity for hydrophobic motifs that are normally found on the interior of properly folded proteins. The presence of unfolded protein in the ER titrates BiP away from the UPR signalling initiators, allowing signals to be relayed to the effector regions in the cytosol (Schroder and Kaufman, 2005) (see Figure 1-5 bottom panel). IRE-1α and PERK closely resemble each other and have serine/threonine kinase cytoplasmic domains (Bertolotti et al., 2000). ER stress induces lumenal-domain-directed homodimerisation, autophosphorylation and activation of IRE-1α and PERK (see Figure 1-5 Ba and Ca). ATF6 in response to ER stress is transported to the Golgi, where it is cleaved by proteases (S1P and S2P) (see Figure 1-5 Aa and Ab). This releases the N-terminal fragment of ATF6 which translocates to the nucleus and activates transcription of target genes (Ye et al., 2000) (see Figure 1-5 Ac).

Structural analysis has revealed that the lumenal domain of yeast IRE-1α is able to detect unfolded proteins directly (Credle et al., 2005). Despite this, overexpression of BiP attenuates transducer activity suggesting a possible hybrid mechanism of unfolded protein recognition (Kimata et al., 2007). However, this may not apply to mammalian IRE-1α, as a recent study showed that mutant IRE-1α, which is unable to bind BiP, is active in the absence of unfolded protein (Oikawa et al., 2009). ATF6 has also been reported to sense ER stress via an alternative mechanism, possibly in response to nutrient

deprivation. Glycosylated ATF6 interacts with calreticulin, anchoring ATF6 to the ER, whereas underglycosylated ATF6 traffics to the Golgi (Hong et al., 2004a). This links the UPR to ER quality control strategies mediated by calnexin and calreticulin (reviewed in (Kleizen and Braakman, 2004)). These alternative mechanisms of sensing unfolded protein may also explain the selective activation of the branches of the UPR seen in certain instances for example plasma cell differentiation (discussed further 1.4.4) (Ma et al., 2010).

1.3.1 PERK

The PERK dependent pathway of the UPR is activated most rapidly in response to ER stress and mediates translational repression via the phosphorylation of eukaryotic initiation factor-2α (eIF-2α) (Harding et al., 1999) (see Figure 1-5 Ca). This immediate response acts to prevent any further accumulation of protein in the ER. This causes a reduction in the level of proteins that are continually expressed and turned over, for example cyclin D1, resulting in G1 cell-cycle arrest in cells experiencing ER-stress (Tomida et al., 1996). However, this translational arrest is not complete, for example, the translation of ATF4 actually requires eIF-2α phosphorylation and ATF4 is important for the recovery from several cellular stresses (see Figure 1-5 Cb). (Harding et al., 2000) (Harding et al., 2003) (Lu et al., 2004b) (Clemens, 2001). ATF4 upregulates genes involved in amino acid synthesis, redox metabolism (Harding et al., 2003) as well as the growth-arrest DNA damage gene 34 (GADD34), which enhances the dephosphorylation of eIF-2α by protein phosphatise 1 (PP1). ATF4 can thereby act as a negative regulator of PERK-mediated inhibition of translation, allowing the expression of the proteins required for ER recovery (see Figure 1-5 Cc) (Novoa et al., 2003). ATF4 and ATF6 also drive the expression of the proapoptotic transcription factor, CCAAT/enhancer binding protein, homologous protein (CHOP), which inhibits transcription of Bcl-2 (Zinszner et al., 1998). This factor may play an important role in switching the UPR from prosurvival to apoptotic signalling (Rutkowski et al., 2006). Finally, PERK is also responsible for the phosphorylation and activation of NF-E2-related factor-2 (NRF2), which acts on genes important for maintaining the redox homeostasis of the ER (Cullinan and Diehl, 2004).

1.3.2 ATF6

The second wave of the UPR is mediated by ATF6, of which there are two paralogues in mammals, ATF6α and ATF6β (Haze et al., 1999). ATF6β has a minimal role in the UPR (Thuerauf et al., 2004). ATF6α is a type II transmembrane protein, containing a cytosolic bZIP transcription factor domain and therefore has the potential to homo- and hetero-dimerise with other members of the bZIP transcription factor family (Newman and Keating, 2003). ATF6α activates transcription from the promoter of genes containing ER-stress response elements (ERSEs) (see Figure 1-5 Ac). These genes include chaperones required for efficient protein folding, for example BiP and protein di-sulphide isomerase (PDI) that increase the folding capacity of the ER as well as genes involved in ER-associated protein degradation (ERAD) (Okada et al., 2002) (Yamamoto et al., 2007). ERAD is the process of retro-translocation of unfolded proteins from the ER lumen into the cytosol for degradation by the proteasome. ATF6 has also been reported to play a role in ER expansion and lipid biosynthesis (Bommiasamy et al., 2009).

Several tissue specific regulators of the UPR have been identified with homology to ATF6. These ER-anchored transcription factors are likely to be activated in a similar manner to ATF6, and integrate the function of the UPR into a range of physiological systems. For example, liver-specific cyclic-AMP responsive element binding protein - hepatocyte (CREBH) is regulated by ER-stress mediated proteolysis but does not act as a typical UPR-transactivator (Omori et al., 2001). CREBH and ATF6 function to activate the systemic inflammatory component of innate immunity, the acute phase response (APR), and therefore link ER stress to liver secretion of inflammatory proteins and the innate immune system (Zhang et al., 2006).

1.3.3 IRE-1

ER stress leads to the activation of both the kinase and endonuclease domains of the ubiquitously expressed IRE-1α isoform (reviewed in (Hetz and Glimcher, 2009)). IRE-1α can then bind to the tumour-necrosis factor (TNF)-receptor-associated factor-2 (TRAF2) which activates JNK signalling via apoptosis signal-regulating kinase-1 (ASK-1) (Urano et al., 2000) (Nishitoh et al., 2002) (see Figure 1-5 Bd). This signalling can activate autophagy, a survival

mechanism that can remove damaged organelles and aggregated proteins (Ogata et al., 2006) (Bernales et al., 2006). JNK activation can also induce apoptosis of those cells that are unable to recover from ER stress, an effect mediated by caspase 12 (Yoneda et al., 2001). IRE-1α activity is also modulated by BAX, BAK and 'BH3 only', members of the B-cell lymphoma 2 (Bcl-2) family of cell death regulators, again connecting the UPR to apoptosis (Hetz et al., 2006) (Klee et al., 2009). These proteins are thought to act as determinants of cell fate depending on the level of ER stress experienced. Severe ER stress leads to a conformational change of these proteins, calcium release from the ER and subsequent cytochrome C release from mitochondria, initiating the caspase cascade resulting in apoptosis (reviewed in (Rao et al., 2004)). A protein tyrosine phosphatase 1B (PTP-1B) is also involved in IRE-1α function. Absence of PTP-1B reduces ER-stress induced apoptosis, JNK activation and XBP-1 splicing (Gu et al., 2004). IRE-1α also modulates other 'alarm sensing' pathways including p38, ERK and NF-κB (Nguyen et al., 2004) (Hu et al., 2006). The outcome of IRE-1α activation is thought to be 'fine-tuned' by its association with different adaptors and modulatory proteins, outlined above. This signalling platform has been referred to as the 'IRE-1α interactome' or UPRosome, and its function depends on the cell type and the nature of the stress encountered (Hetz and Glimcher, 2009).

Recently, a direct role for IRE-1α in the degradation of mRNA has been described as part of an attempt to reprogramme translation towards UPR-induced genes (Hollien et al., 2009) (Han et al., 2009). This function of the endoribonuclease domain of IRE-1α has been proposed to relieve the ER of proteins that are challenging to fold, but may also target mRNA that encodes chaperones and can lead to apoptosis. This highlights the biphasic response of the UPR; firstly to attempt recovery and secondly to mediate cell death where homeostasis cannot be restored.

The complete transcriptional response mediated by IRE-1α is delayed in comparison to that of ATF4 and ATF6 (Yoshida et al., 2003). This is due to the low expression of the substrate for the IRE-1α endonuclease domain, X-box binding protein 1 (XBP-1) mRNA (Nekrutenko and He, 2006). The expression of XBP-1, a bZIP transcription factor, is only increased in response to ER-stress

partly via the ATF6 pathway (Yoshida et al., 2001) (Lee et al., 2002) (Yamamoto et al., 2007). Therefore, after the induction of the initial phase of the UPR, IRE-1α is able to cleave a 26 nucleotide unconventional intron from XBP-1 mRNA (see Figure 1-5 Bb). XBP-1 mRNA is thought to be targeted to IRE-1α by XBP-1 unspliced (XBP-1u) protein, which associates with the ER membrane and recruits XBP-1u transcript (Yanagitani et al., 2009) IRE-1α mediated splicing results in a open reading frame-shift and a transcript that encodes a 371 amino acid, 54 kDa, spliced XBP-1 (XBP-1s) bZIP isoform that has a potent C-terminal transactivation domain (Calfon et al., 2002) (Lee et al., 2002) (see Figure 1-5 Bc).

1.3.3.1 XBP-1

XBP-1 is a critical effector of the UPR and regulates many of the genes required for ER homeostasis, including chaperones and components of ERAD, and is also involved in ER membrane biogenesis (Lee et al., 2003b) (Shaffer et al., 2004) (Sriburi et al., 2004). One of the targets of XBP-1, $p58^{IPK}$, is a negative regulator of PERK-mediated translation repression; highlighting the crosstalk between the UPR axes (see Figure 1-5 Bc) (Yan et al., 2002). This cross talk is also evident in the bZIP heterodimerisation between XBP-1s and ATF6: with many of their target genes overlapping (Newman and Keating, 2003) (Lee et al., 2003b) (Yamamoto et al., 2007). Unspliced XBP-1 (XBP-1u) mRNA is translated to yield a 261 amino acid, 33 kDa, XBP-1u isoform which lacks the transactivation domain and undergoes rapid degradation (Lee et al., 2003a) (Tirosh et al., 2006). It has been reported that XBP-1u may act as a dominant negative regulator of XBP-1s by occupying the binding sequences in target promoters; therefore degradation of XBP-1u is required for XBP-1s activity (Lee et al., 2003a). XBP-1u also accumulates during the recovery phase of ER stress and has been shown to inhibit XBP-1s nuclear entry (Yoshida et al., 2006). XBP-1u therefore acts to negatively regulate the spliced active form (XBP-1s) and this branch of the UPR.

XBP-1 was first identified by its ability to bind to a conserved transcriptional element, the x-box, of the genes encoding MHC class II human leukocyte antigen (HLA) DPB and DRA (Liou et al., 1990). XBP-1 was found to bind preferentially to this promoter element, which is related to the cyclic AMP-

response element (CRE), in additional genes (Clauss et al., 1996). In these XBP-1 cis-acting elements the 'ACGT' core sequence was shown to be highly conserved (Yamamoto et al., 2004) (Kanemoto et al., 2005). Recently, a ChIP-on-chip analysis of XBP-1 binding sites *in vivo* confirmed the targets that contain 'ACGT' (unfolded protein response elements (UPREs)) but also identified related but distinct XBP-1 responsive sequence motifs (Acosta-Alvear et al., 2007). Genes containing the distinct binding motifs belonged to diverse functional categories including cell differentiation and DNA–damage pathways. This study also suggested that XBP-1 may regulate genes in a cell-type dependent manner, possibly by the recruitment of various accessory factors.

Studies using knockout mice have shown that XBP-1 is essential for hepatogenesis, as XBP-1-/- embryos die at day 13.5-14.5 with severe liver hypoplasia (Reimold et al., 2000). In XBP-1-/- mice with a targeted expression of XBP-1 in the liver, defects are reported in the secretory exocrine glands, such as the salivary glands and the pancreas. These animals die early from a failure to secrete digestive enzymes from the acinar cells of the pancreas (Lee et al., 2005). XBP-1 has also been implicated in cardiac myogenesis (Masaki et al., 1999).

Most recently XBP-1 has been found to be important in protecting the cell from the ER stress associated with activation of the innate immune system, auto-immunity and inflammation (Todd et al., 2008). In addition a TLR mediated mechanism of XBP-1 activation in macrophages has been demonstrated which did not involve the induction of the complete ER-stress response (Martinon et al., 2010). However, the outcome of increased bacterial burden and decreased production of inflammatory cytokines when XBP-1 deficient mice are challenged with bacterial infection, does suggest a role for a specific predominantly XBP-1 mediated UPR. Removal of XBP-1 also leads to disrupted ER morphology and increased larval lethality in C.elegans challenged with pathogenic bacteria (Richardson et al., 2010). Further, the risk of developing both types of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) has been associated with several single-nucleotide polymorphisms (SNPs) within the XBP1 gene locus (Kaser et al., 2008). XBP-1 is also important in the development of both conventional and plasmacytoid dendritic cells (DCs)

(Iwakoshi et al., 2007). Spliced XBP-1 was also found in freshly isolated DCs and DC cell lines; indicating the UPR is required for DC function, possibly to facilitate antigen processing and cytokine production. In all these instances, XBP-1 promotes cellular survival, differentiation and function by regulating the folding capacity of the secretory apparatus to allow increased protein synthesis (Shaffer et al., 2004) (Sriburi et al., 2007). For this reason, XBP-1 is vital for the development of professional secretory cells including antibody-secreting plasma cells (see section 1.4.4) (Reimold et al., 2001) (Iwakoshi et al., 2003).

Alongside its function in normal cell development, XBP-1 has also been shown to be necessary for cell survival under hypoxic conditions and tumour growth (Romero-Ramirez et al., 2004); indicating the importance of XBP-1 and the UPR in a variety of physiological stresses that induce ER stress (see section 1.5.2). KSHV is known to infect B-cells and reactivate in response to hypoxia (see section 1.1.6) (Davis et al., 2001). Therefore, this thesis investigates further KSHV reactivation in reaction to the cellular stress associated with B-cell development and hypoxia (see sections 1.4 and 1.5).

1.4 B-cell development

B-cell development can be divided into two stages: the antigen-independent phase that occurs in the bone marrow, to produce mature B-cells (involving the pro-B-cell and pre-B-cell stages), and an antigen dependent differentiation phase that is completed in the periphery (resulting in memory or effector B-cells) (see Figure 1-6) (reviewed in (Johnson et al., 2005b)). Each stage is influenced by external factors and is regulated by a complex transcription factor network (reviewed in (Schmidlin et al., 2009)). Pro-B-cells derive from pluripotent hematopoietic stem cells in the bone marrow, and are stimulated to proliferate by various factors, including stromal cell-derived growth factor-1 (SDF-1) and IL-7, secreted by surrounding cells. Commitment to the B-cell lineage depends on several transcription factors, early B-cell factor, Pu.1, E2A and paired box protein 5 (PAX5) as well as microRNA expression (reviewed in (Busslinger, 2004)) (Chen et al., 2004).

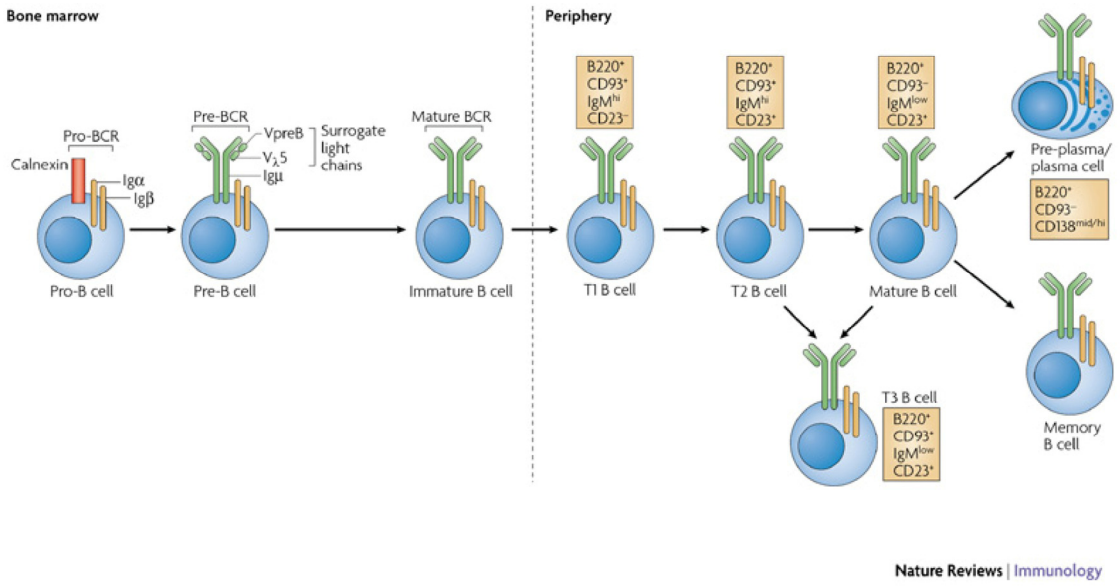

Figure 1-6 B-cell development
B-cell development occurs in both the bone marrow and peripheral lymphoid tissues such as the spleen. In the bone marrow, development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. During this differentiation, rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-B-cell receptor (pre-BCR, which is comprised of an Ig heavy chain and surrogate light chains (VpreB or V5)) and finally a mature BCR (comprised of rearranged heavy- and light-chain genes) that is capable of binding antigen. At this immature stage of development, B cells undergo a selection process to prevent any further development of self-reactive cells. Both receptor editing and clonal deletion have a role at this stage. Cells successfully completing this checkpoint leave the bone marrow as transitional B cells, eventually maturing into mature follicular B cells (or marginal-zone B cells). Following an immune response, antigen-specific B cells develop into either plasma (antibody-secreting) cells or memory B cells. Taken from (Cambier et al., 2007).

B-lymphocytes are part of the adaptive immune system and provide protection against a variety of pathogens; by recognising a broad range of foreign proteins via a diverse B-cell receptor (BCR) repertoire. Diversity is generated early in development, firstly by random rearrangement of immunoglobulin heavy chain (IgH) locus, during the pro-B-cell stage. The process is known as V(D)J recombination and is catalysed by recombinase-activating genes (RAG1/2) and terminal deoxynucleotide transferase (TdT) (reviewed in (Bassing et al., 2002)). Successful recombination of IgH leads to pre-B-cells that express the pre-B-cell receptor (pre-BCR, which is comprised of an Igμ heavy chain and surrogate light chains (VpreB or Vλ5)). This step is necessary for B-cell maturation and allows clonal expansion and rearrangement of light gene (IgL) segments to form an immature BCR that can bind antigen (Ollila and Vihinen, 2005). At this stage, negative selection occurs through tolerance mechanisms including deletion, or receptor editing (reviewed in (Meffre and Wardemann, 2008) (von Boehmer and Melchers, 2010)). This ensures B-cells expressing self-reactive BCRs do not enter circulation thereby preventing autoimmunity and related diseases.

Naive B220+, IgM+ B-cells continue their maturation in the spleen, through a series of 'transitional' stages (T1 and T2) and another round of negative selection, resulting in possible anergy (Chung et al., 2003) (Cambier et al., 2007). T1 B-cells have yet to acquire the ability to re-circulate and are found in the bone marrow and spleen. T2 have entered the follicles and can re-circulate; they have also acquired cell surface IgD and CD23, but still carry markers of immaturity. At this point, in the murine system, a subset of B-cells, termed marginal zone (MZ) B-cells, is derived (reviewed in (Pillai et al., 2005)). In humans these cells circulate in a similar manner to memory cells and secrete 'natural antibodies' without antigenic stimulation; whilst also responding to bacterial and self-antigens (see section 1.4.3) (Martin and Kearney, 2000) (Martin et al., 2001). These cells also have elevated levels of the transcription factor B-lymphocyte induced maturation protein-1 (BLIMP-1), allowing rapid differentiation to plasmablasts. Entry to the MZ B-cell subset in mice is based on low affinity BCR signalling and requires notch and NF-κB signalling (Pillai and Cariappa, 2009). However, in humans it still remains to be determined whether MZ B-cells are a separate B-cell subset or a specialised antigen-specific branch of memory B-cells (see section 1.4.3).

Another minority B-cell subset capable of secreting 'natural antibodies' are the B1 B-cells (reviewed in (Berland and Wortis, 2002) (Shapiro-Shelef and Calame, 2005)). B1 B-cells are also capable of responding to bacterial antigens and have a BCR repertoire that is biased towards recognition of T-cell independent type II antigens and self-antigens (Martin et al., 2001). These cells are predominantly found in the pleural and peritoneal cavities and the gut lamina propria. Although they secrete antibody in a BLIMP-1 dependent manner, they are different from the majority of conventional B-cells in various ways including cell surface markers and an ability to self-renew (Fairfax et al., 2007). It is not known when B1 B-cells diverge from conventional B-cell development and in humans their existence is heavily debated.

The majority of B-cells mature into naive follicular/B-2 conventional B-cells and will be the focus of this introduction. B2 B-cells circulate between the follicles in

the spleen, lymph nodes and bone marrow until they die or encounter their cognate antigen.

1.4.1 B-cell activation

The activation of antigen-specific B-cells requires two signals in order to prevent inappropriate activity: the first is BCR signalling; initiated by encountering antigen, usually presented by antigen-presenting cells (APCs). B-cells meet and respond to antigen through many different mechanisms depending on the cellular context and location in which antigen presentation occurs. This provides the greatest protection from pathogens for the host, as an appropriate response can be initiated to a particular antigen, for example cell-mediated presentation affords greater regulation and coordination of the immune response (reviewed in (Batista and Harwood, 2009)).

B-cell activation is dependent on the overall binding strength or avidity for the antigen; this is increased by the formation of clusters of BCRs (BCR microclusters), to allow successful BCR stimulation (Harwood and Batista, 2008). BCR signalling is propagated further by the spreading of B-cell surface, mediated by extension of lamellipodial protrusions, this allows the B-cell to engage with more antigen in receptor complexes (Fleire et al., 2006) (Harwood and Batista, 2008). BCR activation recruits a range of signalling molecules, including the kinases LYN and spleen tyrosine kinase (SYK), to form a 'signalosome', which can trigger a variety of processes including BCR mediated internalisation of antigen (reviewed in (Harwood and Batista, 2008)). The antigen is then processed and presented on the cell surface in a complex with MHC II (Lanzavecchia, 1985) (Batista et al., 2001). This complex is recognised by the T-helper cell population that have been clonally expanded by recognition of the same antigen; in the formation of an immunological synapse (IS) with an APC (reviewed in (Friedl et al., 2005) (Klein and Dalla-Favera, 2008)). T-cell receptor (TCR) engagement with MHC II on the B-cell, via a second IS, provides the second signal required for B-cell proliferation and differentiation (Lanzavecchia, 1985). Co-stimulatory signals are also required to modulate the B-cell activation response, and occur between B-cell-surface receptors and ligands on T cells and/or APCs (reviewed in (Bishop and Hostager, 2001)). One

of the key interactions is between the tumour-necrosis factor (TNF)-receptor family member CD40, which is expressed by all B cells, and its ligand CD154 expressed by T-helper cells. Upon activation B-cells can either differentiate to form extra-follicular plasmablasts, required for rapid antibody production and early protective immune responses or, with T-cell help, enter a germinal centre (GC) reaction.

1.4.2 Germinal centres

Activated proliferating B-cells can form a germinal centre (GC), which displaces resting B-cells within the follicles of peripheral lymphoid tissues, into a surrounding mantle zone. B-cells within this secondary follicle structure undergo affinity maturation, resulting in the production of both plasma cells, capable of producing high-affinity antibodies, and long-lived memory B cells, which provide protection against secondary antigen challenge (reviewed in (Schmidlin et al., 2009) (Klein and Dalla-Favera, 2008) (Wolniak et al., 2004)). Rapidly proliferating cells known as centroblasts pack densely together to form the dark zone of the germinal centre. These cells undergo the process of somatic hypermutation (SHM), which modifies the genes encoding the immunoglobulin variable regions of the heavy and light chains (reviewed in (Liu and Schatz, 2009)). This is achieved by introducing base pair mutations as well as small deletions and insertions into V(D)J recombination sites (Goossens et al., 1998). This leads to either an increased or decreased affinity for the given antigen. High affinity centroblasts then differentiate into non-dividing centrocytes which migrate to the light zone of the germinal centre. The light zone contains follicular dendritic cells (FDCs), T-cells and macrophages which aid the continued selection for enhanced binding to the antigen. At this stage, and at other times outside of the germinal centre, class switch recombination (CSR) may occur (Toellner et al., 1996). This allows the immunoglobulin isotype expressed by a B-cell to be changed from IgM or IgD to IgA, IgG or IgE; via non-homologous end-joining recombination between specific switch regions upstream of the heavy chain constant regions (reviewed in (Stavnezer et al., 2008)). This alters the function of an antibody without modifying the variable region. This process is linked to SHM; both require the enzyme activation induced cytidine deaminase (AID) to increase antibody diversity (reviewed in (Longerich et al., 2006) (Hackney et al., 2009)). Uracil DNA-glycosidase (UNG) is also required

for CSR (Rada et al., 2002). Centrocytes and centroblasts cycle between the light and dark zones of the germinal centre, clonally expanding and undergoing further rounds of CSR and SHM, in a process known as affinity maturation. Eventually, a centrocyte is antigen selected to differentiate into a memory B-cell or a plasmablast (pre-antibody secreting plasma-cell) (Allen et al., 2004).

The signals required for the differentiation of GC-B-cells are relatively unclear. Differentiation into a memory cells appears to randomly occur throughout the germinal centre reaction (Blink et al., 2005). In contrast post-GC differentiation into plasma cells is driven by a high affinity BCR (Phan et al., 2006). The process is however governed by transcription factors, and their regulation has been shown to control B-cell development. B-cell lymphoma-6 (BCL6) is the master transcriptional regulator of centroblasts and is expressed in some centrocytes (Cattoretti et al., 1995) (reviewed in (Klein and Dalla-Favera, 2008)). B-cells that lack BCL-6 expression do not form germinal centres or undergo affinity maturation (Dent et al., 1997) (Ye et al., 1997). BCL-6 functions as a repressor and downregulates those genes involved in growth suppression and apoptosis, especially in response to genotoxic stress (Polo et al., 2004). This allows the cells of the germinal centre to rapidly proliferate and undergo the necessary processes of SHM and CSR (Shaffer et al., 2000) (Parekh et al., 2007). BCL-6 also has a role in suppressing the genes required for T-cell dependent B-cell activation, preventing premature differentiation of cells (Shaffer et al., 2000). Finally, BCL6 (in combination with Spi-B, an Ets transcription factor) specifically blocks plasma cell differentiation by repression of positive-regulatory-domain-containing-1 (Prdm1), which encodes B-lymphocyte induced maturation protein-1 (BLIMP-1) a repressor crucial for plasma cell development (see Figure 1-7) (Tunyaplin et al., 2004) (Schmidlin et al., 2008) (Shapiro-Shelef et al., 2003) (reviewed in (Crotty et al., 2010)). The metastasis-associated 1 family member 3 (MTA3) is required for BCL6-mediated repression of BLIMP-1 (see Figure 1-7). Removal of this protein using siRNA leads to de-repression of BLIMP-1 and plasma cell differentiation. Interestingly, overexpression of BCL-6 in multiple myeloma (a tumour of plasma cells) results in MTA3 dependent de-differentiation, with cells becoming more GC-like (Fujita et al., 2004).

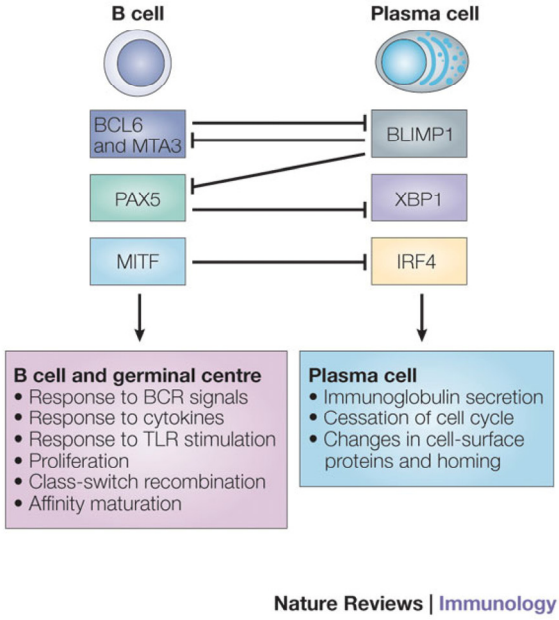

Figure 1-7 Transcription factors control of plasma cell development
Transcriptional repression enforces mutually exclusive B-cell and plasma-cell gene-expression programmes. Several transcription factors — BCL6 (B-cell lymphoma 6), MTA3 (metastasis-associated 1 family, member 3), MITF (microphthalmia-associated transcription factor) and PAX5 (paired box protein 5) — repress plasmacytic development by repressing BLIMP1 (B-lymphocyte-induced maturation protein 1), XBP-1 (X-box-binding protein 1) and IRF4 (interferon-regulatory factor 4). In plasma cells, BLIMP1 represses B-cell gene-expression programmes. This mutual repression prevents the unelicited formation of plasma cells in the germinal centre and prevents the reversion of plasma cells to a B-cell stage. BCR, B-cell receptor; TLR, Toll-like receptor. BCL-6, MTA3, PAX5 and MITF also regulate the expression of genes that are required for B-cell and germinal-centre functions, which are outlined in the pink box. BLIMP1, XBP1 and IRF4 induce the expression of genes that are required for plasma cells, which are outlined in the blue box. Taken from (Shapiro-Shelef and Calame, 2005).

In order for GC-B-cells to growth arrest, interact with other cells and differentiate, BCL6 must be down regulated. BCR signalling has been shown to cause BCL6 degradation via the proteasome. However, BCR-mediated BCL6 repression is not sufficient to induce plasma cell differentiation and many other signals are required to alter the B-cell transcriptional profile (Schliephake and Schimpl, 1996) (Shapiro-Shelef and Calame, 2005) (see section 1.4.4). B-cell engagement with T-cells, and consequent CD40 induced signalling, also inhibits BCL6 transcription by the upregulation of another transcription factor involved in plasma cell differentiation, IRF4 (see Figure 1-7) (Niu et al., 1998) (Basso et al., 2004) (Falini et al., 2000) (Saito et al., 2007) (see section 1.4.4). Cytokines play an important role in guiding the post-GC differentiation, for example the presence of IL-4 and IL-5 increases the potential for plasma cell development (Hasbold et al., 2004). Toll-like receptor (TLR) signalling also plays an important part in B-cell differentiation, with TLR-9-mediated activation of naive B-cells

triggering plasma cell development (Huggins et al., 2007). Finally, activation of signal transducer and activator-3 (STAT3), via IL-21, has been shown to initiate B-cell terminal differentiation via the upregulation of plasma cell genes, such as BLIMP-1 (Ettinger et al., 2008) (Diehl et al., 2008). Therefore although the exact cues for GC exit are unknown, BCL6 degradation terminates the GC transcriptional program, allowing changes in the transcription factor repertoire for post-GC differentiation into long-lived memory or plasma B-cells (see Figure 1-7).

1.4.3 Memory cells

Memory B-cells are long-lived, non-dividing cells that can rapidly proliferate and differentiate into plasma cells in response to secondary encounter with the initial immunising antigen (reviewed in (Tangye and Tarlinton, 2009)). In addition, memory B-cells can respond to polyclonal stimulation through pattern recognition and T-cell help (Bernasconi et al., 2002). Memory B-cells also function to replenish the long-lived antibody-secreting plasma cells (reviewed in (McHeyzer-Williams and McHeyzer-Williams, 2005)). All memory B-cells are IgD^-, are slightly larger than naive B-cells, express higher levels of co-stimulatory and activation markers and the majority express CD27. Memory B-cells can be divided in to various subsets based on the isotype expressed, which has implications for their role in immunity (reviewed in (Sanz et al., 2008)). For example, IgM+ CD27+ B-cells exclusively act against polysaccharide T-independent pathogens and may represent the human counterpart of murine MZ-B-cells (see section 1.4) (reviewed in (Tangye and Tarlinton, 2009)). Another distinct subset expresses the inhibitory receptor Fc-receptor like 4 (FCRL4), and is restricted to mucosal tissues and mesenteric lymph nodes (Ehrhardt et al., 2005). $CD27^-$ B-cells have also been detected in the spleen, tonsils and peripheral blood, however detailed functional analysis of these groups has yet to be undertaken (reviewed in (Tangye and Tarlinton, 2009)). Various mechanisms facilitate the rapid response of memory cells to antigen and include strategic location and a high affinity for the antigen (reviewed in (Schmidlin et al., 2009) (Tangye and Tarlinton, 2009)). In addition, the mechanisms by which memory cells can persist for more than 50 years in humans remains a subject of intense study, but the high level expression of

anti-apoptotic factors is thought to have a role (Good et al., 2009) (reviewed in (Tangye and Tarlinton, 2009)).

The development of memory B-cells requires the germinal centre reaction and memory cells have mutated IgV genes (Tarlinton, 2006). Despite this, the role of BCL6 in this process is not clear. BCL6 is thought to encourage memory B-cell development by blocking plasma cell differentiation. External stimuli have also been shown to be important in regulating memory B-cell differentiation by upregulating BCL6 expression, for example via STAT5 phosphorylation (Scheeren et al., 2005). However, memory B-cells can develop in BCL6 deficient mice, in the absence of GCs (Toyama et al., 2002) and BCL6 over expression was shown to maintain the GC-B-cell phenotype, preventing memory B cell differentiation *in vitro* (Kuo et al., 2007). Therefore, the transcriptional control of B-cell differentiation is complex and involves the interplay between many transcription factors. PAX5 encodes the B-cell specific activator protein (BSAP), another transcriptional repressor essential for maintaining mature B-cell identity (Nutt et al., 1999) (see section 1.4); it therefore must also be downregulated to facilitate B-cell terminal differentiation. PAX5 is expressed in memory B-cells and when PAX5 expression is blocked, in B-cell lines, the plasma cell differentiation transcriptional program is upregulated (Nera et al., 2006). PAX5 is able to directly repress XBP-1 and Ig expression preventing plasma cell development (Reimold et al., 1996) (Singh and Birshtein, 1996) (see Figure 1-7). Continued CD40 signalling has also been implicated in directing centrocytes towards memory B-cell differentiation (Arpin et al., 1995).

1.4.4 Plasma cells and the UPR

The effector cell of the B-cell lineage is the plasma cell, which produces large amounts of immunoglobulin required for both innate and adaptive immunity (reviewed in (Shapiro-Shelef and Calame, 2005) (Radbruch et al., 2006)). BLIMP-1 a five zinc-finger motif containing transcriptional repressor protein is the master regulator of plasma cell differentiation. BLIMP-1 functions to downregulate BCR signalling, arrest the cell cycle and inhibit the germinal centre reaction allowing plasma cell differentiation (Turner et al., 1994) (Lin et al., 1997) (Shaffer et al., 2002). This role was revealed in studies where exogenously supplied BLIMP-1 was sufficient to induce plasmablast

differentiation, while BLIMP-1 conditional knock mice were unable to develop plasma cells (Turner et al., 1994) (Shapiro-Shelef et al., 2003). BLIMP-1 can directly repress BCL6 and PAX5 expression, which alleviates their repression of BLIMP-1 and XBP-1 respectively; amplifying BLIMP-1 expression and allowing Ig production (Shaffer et al., 2002) (Lin et al., 2002) (Tunyaplin et al., 2004) (see Figure 1-7). BLIMP-1 was subsequently shown to act upstream of the transcription factor XBP-1, which is required for plasma cell development (Shaffer et al., 2004) (Reimold et al., 2001) (see section 1.3.3.1). However, overexpression of XBP-1 in the BLIMP-1-/- background failed to restore expression of the marker of plasma cells, CD138 (syndecan-1) and IgM secretion (Shapiro-Shelef et al., 2003). In fact many of the genes upregulated during plasma cell differentiation are upregulated by XBP-1 and BLIMP-1 (Shaffer et al., 2004).

XBP-1 knock-out mice are embryonic lethal (see section 1.3.3.1) therefore, using the recombination activating gene-2 (RAG-2) blastocyst complementation system, chimeric mice were developed. These mice had expected numbers of B-cells that expressed B220+, IgM and IgD, and were able to proliferate in response to CD40 antibody and IL-4 or LPS stimulation (Reimold et al., 2001). These B-cells also express activation markers, undergo CSR and have a normal cytokine profile. However, the levels of Ig secretion are decreased and no antibody response was mounted after challenge with both T-cell dependent and independent antigen, leaving these mice susceptible to polyoma virus infection. Additionally, the B-cells from XBP-1 -/-, RAG-/- chimeric mice did not express CD138 (Reimold et al., 2001).

XBP-1 is essential for the development of Ig secreting plasma cells because of its role in the UPR (see section 1.3.3.1) (Shaffer et al., 2004) (Reimold et al., 2001) (Iwakoshi et al., 2003). Supplying exogenous XBP-1 to XBP-1 deficient B-cells allowed plasma cell differentiation and Ig secretion (Iwakoshi et al., 2003). When XBP-1 is supplied to B-cell lymphoma cell lines that have transcriptional profiles representative of post-GC B-cells or plasmablasts, cells become more plasma cell-like (Shaffer et al., 2004) (Wilson et al., 2007). Gene expression profiling of these cells reveals XBP-1 upregulates genes involved in ER expansion, cell size, lysosome content, mitochondrial mass, ribosome

production, protein synthesis and cellular growth; aspects required to facilitate the synthesis and secretion of a large amount of antibody (Lee et al., 2003b) (Shaffer et al., 2004). Interestingly, BCR signalling has been shown to transiently induce XBP-1 activity and upregulate the expression of several chaperones (Skalet et al., 2005). However, this does not lead to plasma cell differentiation but does indicate that in response to antigen the UPR can be primed, supporting the idea that the UPR is activated prior to antibody production (van Anken et al., 2003). Another model suggests that BLIMP-1 mediated de-repression of Ig secretion leads to ER-stress and IRE-1α activity, which subsequently leads to expansion of the secretory apparatus (Tirosh et al., 2005). Recent studies have shown that XBP-1 activation during B-cell terminal differentiation is a differentiation-dependent event (Hu et al., 2009). This occurs before the UPR is activated and is required after the plasmablast stage of development (Todd et al., 2009). Overall, it is clear that B-cell terminal differentiation requires the transcription factor machinery of the UPR to attain a professional secretory phenotype and produce antibody for the immune response.

IRE-1α is critical for XBP-1 splicing and activity and therefore IRE-1α deficient mice display similar defects in plasma cell development (Zhang et al., 2005). This study also revealed that IRE-1α is required at an earlier stage in B-cell development as IRE-1α-/- B-cells did not progress past the pro-B-cell stage. IRE-1α activity is important for the expression of the enzymes required for Ig gene rearrangement (Zhang et al., 2005) (see section 1.4). ATF6 is cleaved and activated in response to B-cell activation suggesting that this axis of the UPR is also involved in plasma cell differentiation (Gass et al., 2002) (Gunn et al., 2004). The remaining PERK-mediated branch of the UPR network does not appear to have a role in plasma cell development as LPS treatment of PERK-/- B-cells increased their Ig production and developed a specialised secretory phenotype (Zhang et al., 2005) (Gass et al., 2008). Plasma cell differentiation is, therefore, a biological condition that causes selective activation of the IRE-1α and ATF6 branches of the UPR (Wu and Kaufman, 2006) (Ma et al., 2010). However, the question remains as to how this is achieved. PERK activation may be attenuated by $p58^{IPK}$, which is upregulated during the differentiation process, but these cells can still activate PERK in response to further ER stress (Yan et

al., 2002) (Rutkowski et al., 2007) (Gass et al., 2008). During plasma cell differentiation PERK is, therefore, not completely inactivated, suggesting that a threshold level of ER stress is required to activate PERK that may be higher than that of the other UPR branches. Despite PERK and IRE-1α having many similarities, this disparity could also be linked to the ability of the sensors to detect unfolded protein (Bertolotti et al., 2000) (Liu et al., 2000). However, this situation is not true of all professional secretory cells. For example, PERK activation is necessary for the function of pancreatic secretory cells. These cells must deal with periodic increases in the demand for protein synthesis in response to post-parandial increases in blood-glucose (Harding et al., 2001) (Zhang et al., 2002).

The individual axes of the UPR are selectively engaged during physiological stress in a tissue specific manner. Regulation of UPR in these situations is unclear, but probably depends on the formation of regulatory complexes such as the 'UPRosome' as well as interplay between UPR and other signal transduction pathways (see section 1.3.3). Consequently, it is important to consider the contributions of the XBP-1 and UPR in the context of the other known factors that are important for plasma cell differentiation. BLIMP-1 (discussed above) and interferon regulatory factor-4 (IRF4) are the two major transcription factors involved in plasma-cell development. IRF4 is required for mature B-cell formation, and if IRF4 is conditionally knocked-out in GC-B-cells, a defect is also seen in plasma cell differentiation (Mittrucker et al., 1997) (Klein et al., 2006). LPS stimulation of these cells did not lead to antibody secretion and XBP-1 was not expressed. Further study revealed that IRF4 activity is upstream of BLIMP-1 effects (Sciammas et al., 2006). However, evidence also exists that the expression of these genes was independently regulated, at the terminal differentiation stage (Kallies et al., 2007). IRF4 is negatively regulated by MITF, a basic helix-loop-helix transcriptional repressor (see Figure 1-7) (Lin et al., 2004). Knock-down of MITF causes spontaneous B-cell activation and antibody secretion, indicating its involvement in plasma cell differentiation.

Post-transcriptional gene regulation by microRNAs has also been implicated in the regulation of B-cell terminal differentiation. MicroRNA-155 (miR155) is upregulated by antigenic stimulation, and is important in B-cell responses to

thymus-dependent and -independent antigens (Rodriguez et al., 2007). Reduced extra-follicular and germinal centre responses were observed in the absence of miR-155, resulting in reduced IgG1 secretion and impaired affinity maturation (Vigorito et al., 2007). This defect was not due to SHM or CSR but was related to a defect in differentiation and survival of plasmablasts. A role for miR-155 in memory B-cell development was also identified. Amongst the many predicted target genes of miR-155, involved in various functions, the Ets domain-containing transcription factor Pu.1 was confirmed as a direct target. Pu.1 is required at early stages of B-cell development, however its role in terminal differentiation is unclear (McKercher et al., 1996) (Polli et al., 2005). Pu.1 is highly expressed in GC-B-cells and is downregulated in post-GC-B-cells (Cattoretti et al., 2006). Pu.1 downregulation by miR-155 may therefore be important in the output of the GC reaction, but this is yet to be confirmed. Since many other genes, including activation-induced cytidine deaminase (AID), were targeted by miR-155 the phenotypic outcome of miR-155 deletion is probably due to a combination of changes in gene expression (Teng et al., 2008).

1.4.5 B-cell lymphomas

Tumours corresponding to almost all stages of B-cell development have been described in humans, although the majority arise from GC-B-cells (reviewed in (Kuppers, 2005)). The unique physiology of centroblasts allows them to tolerate genotoxic stress (induced by SHM and CSR) and rapidly proliferate, while avoiding apoptosis and cell cycle arrest (see section 1.4.2). For these reasons GC-B-cells are potentially more susceptible to genetic alterations, such as chromosomal translocations and malignant transformation. Tumours maintain many of the characteristics of the cell type from which they are derived. This is clearly evident in B-cell neoplasia where distinctive morphology, immunophenotypes, immunoglobulin gene sequences and recently, gene expression signatures, facilitate classification by their cell of origin (reviewed in (Kuppers et al., 1999) (Shaffer et al., 2006) (Staudt and Dave, 2005)). This is in agreement with the model that malignant B-cells are blocked at otherwise transitory developmental stages (Greaves, 1986). For example, PEL expresses CD138 and IRF4, but XBP-1 is absent, an expression profile representative of a plasmablast cell. PEL cells are therefore blocked prior to terminal differentiation to antibody-secreting plasma cell (see section 1.1.8.2) (Jenner et al., 2003).

Therefore, identification of the particular stage of B-cell development from which the cancer was established is critical for understanding the pathogenesis of the disease, and may lead to the development of therapeutic strategies.

1.4.6 B-cells and gamma-herpesviruses

The interaction between KSHV and its primary *in vivo* target, the B-cell, is not well understood. This is primarily due to the fact that B-cells are relatively refractory to KSHV infection *in vitro*. However, EBV, the most the closely related gamma-herpesvirus to KSHV, readily infects and transforms resting B-cells *in vitro*. Subsequently, the relationship between EBV and human B-cells has been extensively studied (reviewed in (Thorley-Lawson, 2005)). EBV infects naive B-cells *in vivo* and mimics certain aspects of B-cell differentiation to drive the infected cells towards a memory cell phenotype (Souza et al., 2005) (Thorley-Lawson and Babcock, 1999). Establishment of latent EBV infection in the memory B-cell compartment allows lifelong persistence within the host. The information on EBV infection of B-cells allows one to infer possible similarities between the two viruses.

Table 1-5 EBV expression programmes (adapted from (Thorley-Lawson, 2001))

Expression programme		**Proteins expressed**	**Expression observed in**	**Function**
Latency III	Growth programme	EBNA1-6 LMP1 LMP2A-B	Activated B-cell LCLs	Activates resting B-cells to form lymphoblasts
Latency II	Default programme	EBNA1 LMP1 LMP2A	GC-B-cell Classical Hodgkin's lymphoma	Episome maintenance, survival and proliferation
Latency I		EBNA-1 LMP2A transcript	Burkitt's lymphoma	Allows persistence
Latency 0	Latency Programme	None	Memory B-cells	Allows persistence
Lytic replication		Ordered lytic cascade	Plasma cells	Produces infectious virions

EBV like all herpesviruses has a latent and a lytic phase to its life cycle. The latent phase can be further divided into three programmes by transcriptional profiling (see Table 1-5). Upon infection of a naive B-cell EBV expresses the set of latent genes associated with the growth programme (Latency III – see Table 1-5). The expression of these viral gene products is sufficient to activate the B-cell in a similar manner to antigen encounter (reviewed in (Thorley-Lawson, 2005)). Activated B-cells then migrate to the follicle, and the EBV transcriptional profile changes to the default programme in order to drive the B-cell through the GC reaction (Latency II – see Table 1-5). LMP-1 causes Ig gene mutation while LMP-2 induces isotype switching and together they mimic signals caused by antigen encounter and T-cell help, allowing the EBV infected cell to avoid negative selection and survive the GC reaction (see section1.4.2) (Burkhardt et al., 1992) (Kilger et al., 1998). LMP-1 also inhibits BCL6 expression, signalling the B-cell to leave the GC and enter the memory cell pool (Panagopoulos et al., 2004). Circulating EBV infected memory cells are associated with the latency programme (Latency 0/1 – see Table 1-5), where minimal viral proteins are expressed, allowing immune evasion and non-pathogenic persistence. EBV does not drive constant proliferation. Instead, a transient burst of proliferation is required to activate and differentiate cells into resting memory B-cells. However, in an immuno-suppressed environment, when errors occur in this process (i.e. cells do not exit the growth programme) tumours can develop (reviewed in (Thorley-Lawson, 2005)).

EBV reactivation has been linked to plasma cell differentiation via the transcription factor XBP-1 (Laichalk and Thorley-Lawson, 2005) (Sun and Thorley-Lawson, 2007) (Bhende et al., 2007) (McDonald et al., 2010). Antibody secreting cells not only provide the ideal environment (i.e. expanded secretory apparatus) for herpesvirus production but also migrate to the mucosal epithelium, for example the tonsils; a perfect site for shedding virus into the saliva for dissemination.

The stage of development of the B-cell targeted for KSHV infection *in vivo* is not known. Recent in vitro studies have indicated the possible requirement of an activated phenotype (Rappocciolo et al., 2008). However, cells of the KSHV

associated disease, PEL, represent plasmablast cells, a B-cell stage just prior to terminal differentiation (see sections 1.1.8.2 and 1.4). Whether KSHV adopts the EBV model of driving B-cell differentiation remains to be answered. Despite the potential for several KSHV latency programmes, only one gene expression profile is seen to predominate (see section 1.1.12). A connection between KSHV reactivation and plasma cell differentiation has however been reported and is investigated further in this thesis (Wilson et al., 2007) (Sun and Thorley-Lawson, 2007).

1.5 Hypoxia

Oxygen is essential for the survival of complex organisms, and high or low levels can cause cellular stress (reviewed in (Brahimi-Horn and Pouyssegur, 2007)). *In vivo*, hypoxia is functionally defined as occurring whenever oxygen (O_2) demand is greater than supply (reviewed in (Nizet and Johnson, 2009)). Hypoxia causes dramatic changes in cell physiology including cell cycle arrest, anaerobic glycolysis, increased expression of survival and proliferation factors and increased cell motility and invasion (Harris, 2002). Normal oxygen levels can be reduced as a result of tissue damage or other changes to perfusion, and is associated with various patho-physiological conditions including ischemic disorders, diabetes, inflammatory disease, psoriasis and cancer. However, low oxygen conditions also occur during development and are found in normal physiology (see Table 1-6) (Ivanovic, 2009). For example, low oxygen levels found in lymph nodes are important in slowing down lymphocyte migration contributing to cell interaction time (Huang et al., 2007). The hypoxic microenvironment of the bone marrow is also crucial in protecting stem cells from damage caused by oxygen radicals, and may control the expression of genes associated with the self-renewing capacity for example NOTCH and telomerase (Parmar et al., 2007) (Gustafsson et al., 2005).

Physiological oxygen tensions can vary between 2.5-9 % O_2, depending on the organ and its perfusion, classifying many tissues as hypoxic from a normal atmospheric oxygen perspective (see Table 1-6). This highlights the non-physiological conditions that are represented by cell culture at 21 % O_2, regarded as normoxia (hyperoxia - (Ivanovic, 2009)); while hypoxia is routinely taken to be between 0.5-3 % O_2 by volume in the air that perfuses the growth

medium. Anoxia is defined as the absence of physiologically available oxygen and can occur in areas of severe damage and acute inflammation (Nizet and Johnson, 2009). Oxygen has a relatively short diffusion distance of only 100-200 μm and therefore oxygen tensions rapidly reduce with distance from the oxygen supplying blood vessels. For this reason, hypoxia is a common feature of solid tumours and predicts a poor prognosis, the potential for metastasis is increased and the efficacy of treatment is decreased (Hockel and Vaupel, 2001) (Harris, 2002) (Maxwell, 2005).

Table 1-6 Summary of oxygen tensions in mmHg and percentage oxygen content of various conditions.

Condition	**Oxygen tension (mmHg)**	**Oxygen levels (%)**	**Reference**
Atmospheric/tissue culture	160	21	
Upper Airway	150	~20	(Yee Koh et al., 2008)
Lungs	104-150	13-20	(Brahimi-Horn and Pouyssegur, 2007)
Arterial Blood	95	~12	(Arnett et al., 2003)
Venous and Capillary blood	40	5	(Arnett et al., 2003)
Tissue levels (organ specific)	20-70	2.5-9	(Nizet and Johnson, 2009)
Bone Marrow	50	6.6	(Harrison et al., 2002)
Thymus	10	~1	(Braun et al., 2001)
Lymphoid organs	4-35	0.5-4.5	(Caldwell et al., 2001)
Retina	5	~0.5	(Yee Koh et al., 2008)
Hypoxia (HIF-1α stabilisation)	10-22	1-3	(Koumenis and Wouters, 2006)
Wounds and necrotic tissue	<10	<1	(Nizet and Johnson, 2009)

Pathology	<10	<1	(Lewis et al., 1999)
Anoxia	0	0	

1.5.1 Hypoxia inducible factor

Hypoxia inducible factor (HIF) is the master regulator of the molecular adaptive response to hypoxia-induced cellular stress (reviewed in (Weidemann and Johnson, 2008) (Wenger, 2002)). HIF is known to act as a heterodimeric complex consisting of 120 kDa HIF-α and a ~90 kDa HIF-β subunit (Wang et al., 1995). Both subunits are members of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) transcription factor family. HIF induces the transcriptional program essential for returning cells to homeostasis upon exposure to low oxygen conditions (Wang and Semenza, 1993a). This is achieved via HIF-mediated regulation of over a hundred genes involved in diverse processes including angiogenesis, anaerobic glycolysis, mitochondrial oxygen consumption and intracellular pH regulation (Wang et al., 1995) (Manalo et al., 2005) (Semenza et al., 1994) (Firth et al., 1994) (Fukuda et al., 2007) (Shimoda et al., 2006). Due to its functions, HIF also has a crucial role in tumour development and survival (Harris, 2002) (Semenza, 2010) (Maxwell, 2005).

There are three isoforms of HIF-1β (aryl hydrocarbon receptor nuclear translocator-1 (Arnt1) 1, 2 and 3); there are also three HIF-α isoforms (HIF-1α, 2α and 3α. HIF-1α and HIF-2α (also known as endothelial PAS domain protein 1 (EPAS1)) have similar domain structure and are regulated in an analogous manner (Hu et al., 2003) (Wang et al., 2005b). Various studies have demonstrated both non-redundant and overlapping roles for these isoforms in a cell-type dependent manner and therefore further study is required to investigate the target gene specificity (Hu et al., 2007) (Wiesener et al., 2003) (reviewed in (Bardos and Ashcroft, 2005)). However, studies have revealed essential but different roles for HIF-1α and HIF-2α in embryonic development. Targeted disruption of HIF-1α or HIF-2α leads to lethality but at different stages and with variable phenotypes (reviewed in (Huang and Bunn, 2003)). Tissue-specific deletion of HIF has indicated a role, not only in adaptation to hypoxia, but also for physiological function in many cell-types, including a role

haematopoietic differentiation, B-cell development and innate immune responses (Nguyen-Khac et al., 2006) (Kojima et al., 2002) (see section 1.5.3) (Nizet and Johnson, 2009). The role of the final HIF-α isoform, HIF-3α, is not completely clear. However, a product of alternative splicing of this gene removes the transactivation domain. The remaining N-terminal basic helix-loop-helix and PAS domain containing protein (IPAS) can heterodimerise with HIF-1α inhibiting transactivation, providing a negative feedback system of HIF regulation (Makino et al., 2001) (Heidbreder et al., 2003). A natural antisense transcript of HIF-1α (aHIF) has also been identified and may contribute to the post-transcriptional regulation of HIF-1α (Uchida et al., 2004).

HIF was discovered as the transcription factor responsible for the upregulation of the erythropoietin (EPO) gene under hypoxic conditions (Semenza et al., 1991) (Semenza and Wang, 1992). HIF-α and HIF-β (Arnt) subunits are constitutively expressed but HIF-α subunits are rapidly degraded in the presence of oxygen (half-life of <5mins) (Huang et al., 1998) (Salceda and Caro, 1997). This process is regulated by a family of oxygen-, iron- and 2-oxoglutarate-dependent prolyl hydroxylases (PHD1/2/3), which are themselves highly regulated by, among other factors, the presence of reactive oxygen species (ROS), intracellular calcium fluctuations, E3 ubiquitin ligases and HIF (Bruick and McKnight, 2001) (Berra et al., 2006) (Simon, 2006) (Berchner-Pfannschmidt et al., 2004) (Nakayama and Ronai, 2004) (reviewed in (Schofield and Ratcliffe, 2005)). High concentrations of the immuneregulatory molecule nitric oxide (NO) are thought to positively regulate HIF-1α accumulation by inhibiting PHDs (Mateo et al., 2003).

In the presence of oxygen HIF-conserved proline residues (Pro402 and Pro564) within the oxygen dependent degradation domain (ODD) are hydroxylated by PHDs (see Figure 1-8 A). As a consequence the von Hippel-Lindau tumor suppressor (VHL) E3-ligase complex is recruited, facilitating the polyubiquitination and proteasomal degradation of HIFα (see Figure 1-8 B) (Ohh et al., 2000) (Ivan et al., 2001) (Yu et al., 2001) (Jaakkola et al., 2001) (Safran and Kaelin, 2003).

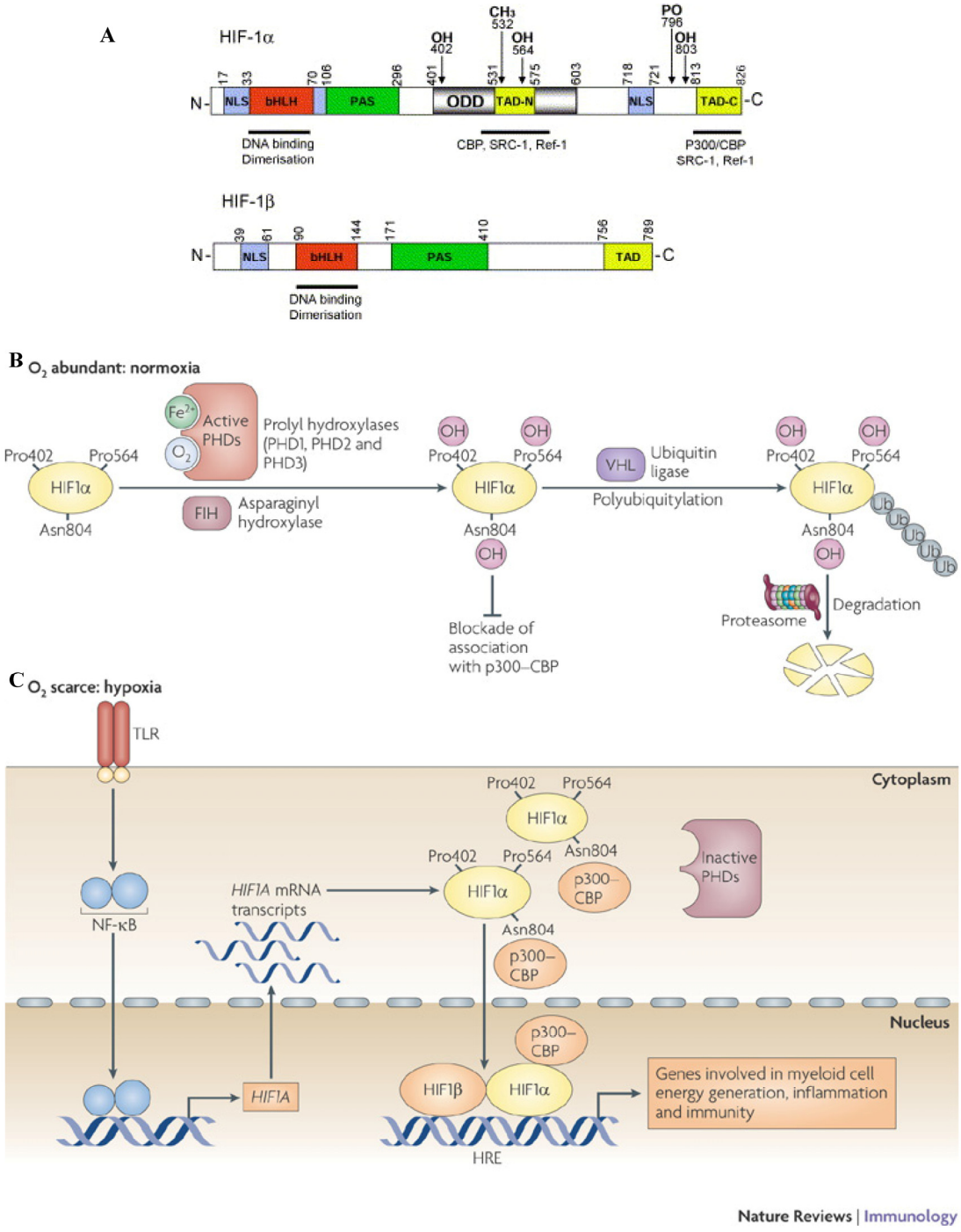

Figure 1-8 Protein domains of HIF-1α and HIF-1β and regulation of HIF-1α
(A) Functional domains and binding domains with co-factors are shown. Hydroxylation, acetylation and phosphorylation sites are indicated. bHLH, basic helix-loop-helix; ODD, oxygen-dependent degradation domain; N-TAD and C-TAD, N- and C-terminal transactivation domain; NLS, nuclear localisation signal; PAS, Per-ARNT-Sim. Taken from (Dery et al., 2005). (B) Under normoxic conditions, hydroxylation of hypoxia-inducible factor 1 (HIF1) by prolyl hydroxylases (PHDs) occurs in an O2-dependent manner at amino acid residues 402 and 564. This results in polyubiquitylation of the HIF-1α protein by von Hippel–Lindau tumour suppressor protein (VHL) and ultimately in the degradation of HIF-1α by proteasomes. The asparaginyl hydroxylase factor inhibiting HIF (FIH) functions in conjunction with the prolyl hydroxylation, although in this case to hydroxylate an asparagine residue in the carboxy-terminal domain of HIF-1α. This blocks the association of HIF-1α with p300–CREB-binding protein (CBP; also known as CREBBP), which in turn inhibits transcriptional enhancement by the HIF complex (not shown). As all of these post-translational events depend on intracellular oxygen they are inhibited by oxygen deprivation. (C) Under hypoxic conditions, HIF-1α gene expression is upregulated through nuclear factor-κB (NF-κB) activation downstream of Toll-like receptors (TLRs). PHDs and FIH

are no longer able to hydroxylate HIF-1α. The unmodified form of HIF-1α in association with p300–CBP migrates to the nucleus to bind HIF-1β (also known as Arnt), forming a heterodimeric helix–loop–helix transcriptional regulator. The HIF complex binds to target promoters known as hypoxic-response elements (HREs). Adapted from (Nizet and Johnson, 2009).

The critical role played by VHL in the regulation of HIF-1α regulation is highlighted in VHL disease, where inactivation of VHL leads to the development of highly vascularised tumours of the kidney, retina and central nervous system (Kaelin, 2007). Therefore, VHL stability and its affinity for the components of the E3 ubiquitin ligase complex and HIF-1α are normally tightly regulated by various mechanisms. These include ubiquitylation and subsequent degradation mediated by ubiquitin carrier protein (UCP), a member of the E2 enzyme family (Jung et al., 2006) (reviewed in (Yee Koh et al., 2008)). VHL mediated degradation of HIF-1α is also dependent on localisation. HIF-1α has been show to accumulate and undergo degradation in different compartments in a cell-type dependent manner (Zheng et al., 2006). Also during acidosis, which occurs in hypoxic tissues, VHL nucleolar sequestration has been observed; adding the further complexity of pH conditions to VHL mediated HIF-1α control (Mekhail et al., 2004).

Transcriptional activity of HIF-α is regulated by the oxygen-, iron- and 2-oxoglutarate -dependent asparaginyl hydroxylase factor inhibiting HIF (FIH), which targets asparagine residues within the C-terminal transcriptional activation domain (C-TAD) of HIF (see Figure 1-8 A) (Lando et al., 2002b). This blocks any stabilised HIF from interacting with coactivators such as the p300-CREB-binding protein, inhibiting co-activation of HIF responsive genes (see Figure 1-8 B) (Lando et al., 2002a) (Wenger, 2002) (reviewed in (Lisy and Peet, 2008)). Under conditions of oxygen or iron deprivation, the prolyl and asparaginyl hydroxylases are unable to hydroxylate the HIFs, which are therefore no longer targeted for degradation (Jewell et al., 2001) (see Figure 1-8 C). The level of HIF-1α stabilisation varies over the physiological range of oxygen tensions, and has been shown to be at its half maximal level between 1.5-2 % O_2 (Jiang et al., 1996b). Stabilised HIF-1α and the 48 % homologous HIF-2α, heterodimerise with the HIF-1β subunit and bind to hypoxia response elements (HREs) (Jiang et al., 1996a). HREs have the core recognition sequence 5'-(T/A)CGTG-3' (Semenza and Wang, 1992) and are present in the

promoter regions of HIF regulated genes for example, glucose transporter 1 (GLUT1) and vascular endothelial growth factor (VEGF) (Firth et al., 1994) (Semenza et al., 1996) (Forsythe et al., 1996) (Harris, 2002). In addition, HIF-1α has also been reported to have a non-transcriptional role in promoting the differentiation of leukemic cells (reviewed in (Zhang and Chen, 2009)).

This mechanism of HIF control by the oxygen-sensing mechanism of hydroxylation is the primary method of regulation. The HIF-mediated response is also negatively and positively regulated by a number of interactions with numerous coactivators or proteins that often lead to post-translational modifications. These interactions are not always dependent on hypoxia and may determine the genes targeted (reviewed in (Dery et al., 2005) (Bardos and Ashcroft, 2005) (Yee Koh et al., 2008) (Lisy and Peet, 2008)). The coactivator hepatocyte nuclear factor 4 (HNF4) is a liver and kidney specific transcription factor required for EPO transcription. HNF4 acts by binding to the EPO promoter at adjacent sites to the HRE and enhancing transcriptional activity of HIF-1α (Zhang et al., 1999). Post-translational modifications of HIF-1α include acetylation by the acetyltransferase ARD1, and sumoylation, both of which promote hydroxyl-proline independent VHL recruitment in normoxia or hypoxia, enhancing HIF-1α degradation (Jeong et al., 2002b) (Cheng et al., 2007). Conversely, S-nitrosylation of HIF-1α cysteine residues under normoxia prevents degradation and is thought to influence the interaction with p300 and therefore HIF-1α transcriptional activity (Li et al., 2007) (Yasinska and Sumbayev, 2003) (Cho et al., 2007). Importantly, increased HIF-1α transcriptional activity is seen in response to MAPK dependent phosphorylation, linking the HIF-1α response to growth factor signalling (Richard et al., 1999) (Sang et al., 2003). In fact normoxic induction of HIF-1α occurs in response to a variety of cytokines and growth factors including TNF-α and insulin (Jiang et al., 2001) (Hellwig-Burgel et al., 1999). The phosphatidylinositol 3-kinase-Akt (PI3K-Akt) pathway has been implicated in upregulating HIF-1α translation in response to growth factors but also in regulation of HIF-1α degradation; its direct role in regulating the response to hypoxia is therefore not clear (reviewed in (Bardos and Ashcroft, 2005)). Glycogen synthase kinase 3 β (GSK3-β), which is inactivated by Akt, also leads to VHL-independent ubiquitination and degradation but this appears to depend on the level and duration of the hypoxia

experienced (Flugel et al., 2007) (reviewed in (Bardos and Ashcroft, 2005)). Analogously, the fork-head box transcription factor, FOXO4, negatively regulates HIF-1α and is inactivated by Akt (Tang and Lasky, 2003).

The association of chaperone proteins, such as heat shock protein 90 (Hsp90), leads to increased HIF-1α stability by disrupting the interaction between HIF-1α and the receptor of activated protein C kinase (RACK1). This prevents the oxygen-independent recruitment of components of the E3 ligase complex and therefore HIF-1α degradation (Liu et al., 2007a) (reviewed in (Yee Koh et al., 2008)). The RACK1-mediated pathway of HIF-1α regulation can also be modulated by calcium via Calcineurin A, which dephosphorylates RACK1 preventing dimerisation and activity (Liu et al., 2007b). Hypoxia leads to an increase in free calcium in the cytoplasm due to an influx in extracellular calcium, combined with the release of calcium from the ER (Seta et al., 2004). Various calcium-dependent mechanisms of HIF-1α regulation have been proposed but this area remains complicated and requires further study (Berchner-Pfannschmidt et al., 2004) (reviewed in (Yee Koh et al., 2008)). Hypoxia also leads to the production of reactive oxygen species (ROS) by mitochondria, which can stabilise HIF-1α (Guzy et al., 2005) (Simon, 2006) (reviewed in (Dewhirst et al., 2008)).

The importance of HIF-1α in tumour progression is highly significant and many cancer inducing mutations further enhance the HIF response (Semenza, 2010). HIF has two major roles in tumorigenesis: i) HIF is required for angiogenesis to facilitate blood supply to the growing tumour and ii) HIF mediates the 'Warburg effect', whereby tumour cells under normoxia have an increased rate of glycolysis compared to normal tissues. HIF is also involved in the increased proliferation, invasion, metastasis as well as apoptosis (reviewed in (Rankin and Giaccia, 2008)). Several tumour suppressor proteins $p14^{ARF}$, PTEN and p53, which are deregulated in many human tumours, have been shown to negatively regulate HIF-1α (reviewed in (Bardos and Ashcroft, 2005)). Oncoproteins such as v-src have also been implicated in the induction of HIF-1α (Jiang et al., 1997a). The central regulator of the inflammatory response, NF-κB has also been shown to increase HIF-1α mRNA production under hypoxic conditions

again linking the hypoxic response to cell function during inflammation (Frede et al., 2006) (see Figure 1-8 C).

HIF-1α protein levels can be regulated by a vast number of mechanisms both dependent and independent of oxygen levels. The final major means of HIF-1α regulation is at the level of translation. Various growth factors, cytokines and oncoproteins have been shown to control HIF-1α protein translation (reviewed in (Yee Koh et al., 2008)). Activation of the PI3K mammalian target of rapamycin (mTOR) and MAPK leads to the formation of the eIF4F complex required for cap-dependent translation. Under hypoxia protein translation is much reduced in order to reduce the energy consumed under stress conditions (Liu et al., 2006) (see section 1.5.2). However, HIF-1α and proteins required for cellular survival continue to be translated despite the mechanism(s) for this being unclear (reviewed in (Yee Koh et al., 2008)). This may include the recruitment of the RNA binding protein, polypyrimidine tract-binding protein (PTB), to enhance translation (Galban et al., 2008).

The HIF-1α mediated response therefore depends on a balance between HIF-1α synthesis, degradation and activity, regulated by both oxygen dependent and independent mechanisms. This highlights the additional roles of HIF-1α outside of the hypoxic response. By having a large number of regulatory mechanisms, the HIF-1α response can be tailored to the conditions experienced, for example prolonged versus transient hypoxia.

1.5.1.1 Hypoxia mimics

Due to the dependence of PHDs and FIH prolyl hydroxylases on iron (Fe(II)) for activity, HIF stabilisation and 'hypoxia' can be mimicked by iron chelators such as desferoxamine and a range of metals including CO(II), Ni(II) and Mn(II) (Schofield and Ratcliffe, 2004) (Wang and Semenza, 1993b) (Goldberg et al., 1988). The addition of compounds such as colbalt chloride inhibits PHDs which consequently leads to the stabilisation of HIF-α protein and the transactivation of HIF dependent genes (Schuster et al., 1989). However, the physiological relevance of hypoxia mimics is questionable as the response to hypoxia is not solely confined to the actions of HIF (reviewed in (Nakayama, 2009)). Numerous studies of gene expression profiles in response to hypoxia have

shown that HIF activation is not responsible for all the changes seen (reviewed in (Feldman et al., 2005)). The activity of major signalling pathways is altered rapidly and reversibly during hypoxia, predominantly in attempt to save energy and maintain cell viability. These pathways include the mTOR pathway, which is inactivated by hypoxia; mediating the inhibition of protein translation and in some instances apoptosis (Arsham et al., 2003) (Hamanaka et al., 2005). The NF-κB pathway is also activated by hypoxia and has a role in promoting cell survival and proliferation (Cummins et al., 2006). Recent work has shown that the expression of a selection of miRNAs is also regulated by hypoxia and contributes to the gene expression changes seen, possibly in a HIF-independent manner (reviewed in (Kulshreshtha et al., 2008) (Ivan et al., 2008)). The direct role for HIF-1α in the upregulation of miRNA expression has however been confirmed for some miRNA including miR-210. The targets of miR210 are still being uncovered, but include the receptor tyrosine kinase Ephrin-A3, important in the endothelial cell response to hypoxia (Fasanaro et al., 2008). Another important pathway activated by hypoxia is the ER stress pathway or UPR discussed further in section 1.5.2.

Therefore in order to investigate the cellular response to hypoxia it is important to use the appropriate conditions. In some instances using hypoxia mimics should be considered as insufficient, as they do not induce the same complex cellular response as low oxygen 'authentic hypoxia' conditions. It is also important to consider the fact that hypoxia is a term given to a range of low oxygen tensions and does not specify the duration of exposure. The cellular response and gene expression pattern seen depends on both the severity and length of hypoxic exposure (reviewed in (Koumenis and Wouters, 2006)). It is therefore critical to obtain the most physiologically relevant hypoxia conditions to those that you wish to investigate (Ivanovic, 2009).

1.5.2 Hypoxia and the UPR

In a study by Romero-Ramirez *et al*. the global changes in gene expression under severe hypoxia were analysed and the UPR genes including those of the PERK and IRE-1/XBP-1 pathways were 'mostly robustly induced' (Romero-Ramirez et al., 2004) (see section 1.3). Work by Koumenis and colleagues went onto show that cells cultured under hypoxic conditions, and transformed cells in

hypoxic regions of solid tumours, activate a translational control program known as the integrated stress response (ISR) (reviewed in (Koumenis and Wouters, 2006) (Liu et al., 2006) (Moenner et al., 2007)). This response is partly mediated via the activation of PERK and its phosphorylation of eIF2-α, causing hypoxia-induced translation attenuation which allows cellular adaptation to ER stress and cell survival (Koumenis et al., 2002). PERK therefore plays a key role in tumour development with PERK-/- tumours displaying slower growth rates and increased apoptosis (Bi et al., 2005). As mentioned previously, translational arrest in response to hypoxia is also mediated by the UPR independent mTOR pathway. Hypoxia inhibits mTOR function through multiple pathways reducing phosphorylation of its target proteins and preventing cap-dependent translation initiation (Brugarolas et al., 2004) (reviewed in (Wouters and Koritzinsky, 2008)).

The cell mounts a biphasic response in order to control mRNA translation, indicating the importance of this HIF-independent reaction to hypoxia (reviewed in (Wouters et al., 2005)). In fact, under severe hypoxia, the ATP demand for protein production is reduced by up to 7 %; aiding adaptation to the low oxygen and energy deficient conditions (Hochachka et al., 1996). However, during this general translational arrest certain genes, including HIF-1α, angiogenic factors and ATF4, are selectively translated and many contribute to hypoxia tolerance (Blais et al., 2006) (Blais et al., 2004). During hypoxia the translational machinery is redirected towards hypoxia-induced mRNA, an effect known as ‘translational reprogramming’ (Ron and Walter, 2007). How this is achieved is still not completely understood, suggested mechanisms include internal ribosome entry site (IRES) elements which facilitate cap-independent translation as well an important role for miRNA (Hua et al., 2006). Importantly, the UPR, mTOR and HIF pathways do not act in complete independence, and a number of interactions have been observed; including negative feedback between the UPR and mTOR pathways, and transcriptional cooperation between the HIF and UPR mediated responses (reviewed in (Wouters and Koritzinsky, 2008)).

Investigation of hypoxic regulation of XBP-1 has shown that low oxygen conditions increases the transcription of XBP-1 and also activates splicing.

Transformed cells deficient in XBP-1 are more sensitive to hypoxia-induced apoptosis, and tumour growth is strongly inhibited compared to wild type cells (Romero-Ramirez et al., 2004). These results indicate that XBP-1 has a crucial role, during hypoxia, in cell survival and the regulation of tumour expansion, and indicates that another mechanism exists to respond to hypoxia other than that mediated by HIFs. As yet hypoxic activation of ATF6, the third ER stress sensor, has not been demonstrated but is highly likely as many of its transcriptional targets are seen to be upregulated under hypoxic conditions.

How the UPR is triggered by hypoxia however is still debated; the rapid inactivation of the translation initiation complex by PERK mediated phosphorylation of eIF2-α suggests that misfolded protein accumulates under low oxygen conditions (Koritzinsky et al., 2006). ER protein folding and maturation involved various steps including chaperones, oligosaccharide modification, isomerisation and di-sulphide bond formation. Oxygen provides the oxidative potential required for di-sulphide bond formation in yeast, however; whether oxygen acts as the crucial electron acceptor in the mammalian ER is yet to be confirmed (Tu and Weissman, 2002) (reviewed in (Tu and Weissman, 2004)). The UPR however is an extremely coordinated cellular response that facilitates the recovery or removal of cells undergoing ER stress and plays a crucial role during hypoxia (see section 1.3). Recently the UPR was also implicated in the upregulation of VEGF-A linking the UPR to angiogenesis, highlighting the importance of energy and oxygen supply for ER function (Ghosh et al., 2010).

1.5.3 Hypoxia and B-cell development

HIF-1α also has a role in B-cell physiology. This is perhaps not surprising as immune cells are exposed to low oxygen tensions as they develop in secondary lymphoid tissues, and migrate between the blood and different tissues (Caldwell et al., 2001) (reviewed in (Sitkovsky and Lukashev, 2005)). HIF-1α has also been shown to be stabilised in the microenvironments of the germinal centres (GCs) (Piovan et al., 2007), secondary lymphoid tissues and bone marrow possibly due to low oxygen availability (Parmar et al., 2007) (see section 1.5.1).

The importance of HIF-1α in B-cell development was highlighted in a study using HIF-1α knock out RAG2-/- chimeric mice. These mice display an increase in appearance of abnormal B-1-like peritoneal lymphocytes, expressing high levels of B220+. An accumulation of autoantibodies were also seen in the serum of these mice as well as a disturbed maturation of the B-2 cell subset in the bone marrow (Kojima et al., 2002). Further studies have shown that the energy supply generated by HIF-1α mediated glycolysis is required for specific stages of B-cell development (Kojima et al., 2010). How HIF-1α is stabilised during B-cell development however is unclear, the microenvironment of the bone marrow has been reported as hypoxic while the insulin-like growth factor (IGF-1), which also has a role in promoting pro-B-cell development can also regulate HIF-1α (Taguchi et al., 2006).

1.6 Aims of this thesis

Understanding the relationship between the virus and its host is fundamental to virus biology, and importantly may provide new insights into the design of novel therapeutic treatments. The switch between latent and lytic KSHV infection is an aspect of the KSHV life-cycle that is not well understood. This thesis focuses on the physiologically relevant, cellular cues for KSHV reactivation and aims to investigate further the interactions between KSHV and B-cells. In particular, we aimed to further develop an *in vitro* cell-based system for investigating the molecular detail of KSHV lytic reactivation. We used this system to determine the mechanism for XBP-1s-induced KSHV reactivation and suggest that this virus-host interaction may be conserved among other related viruses.

As hypoxia can activate the UPR and XBP-1 we hypothesised that the lytic reactivation of KSHV seen in response to hypoxia is caused by the presence of active XBP-1. In order to address this question we aimed to establish authentic ‘low oxygen’ hypoxic tissue culture conditions that cause KSHV reactivation. We then dissect the roles of XBP-1s and HIF-1α in hypoxia-induced KSHV reactivation. Finally, we investigated further the role of XBP-1s in plasma cell differentiation of KSHV infected PEL cells. Focusing on gene expression changes induced during XBP-1s expression in PEL and the subsequent virus lytic replication cycle.

2 Materials and Methods

2.1 Cell culture

All cell culture work was carried out in class II biosafety cabinets using sterile technique. All culture plates and flasks were from Techno Plastic Products (TTP, Switzerland).

B-cell lines used in this study are derived from B-cell tumours, some are infected with KSHV and/or EBV and were grown in complete medium, Roswell Park Memorial Institute (RPMI) - 1640 medium (GIBCO, Invitrogen, Paisley, UK) with 10 % foetal calf serum (FCS, BioSera) and 100 units/ml penicillin plus 100 µg/ml streptomycin (P/S, GIBCO, Invitrogen, Paisley, UK). Cells were split 1:10 to 1:20; depending on density and growth rate twice a week (Table 2-1).

Table 2-1 B-cell lines used in this study

Cell line	*B-cell tumour*	*Viruses*	*Reference*
BC-3	Primary effusion lymphoma	KSHV	(Arvanitakis et al., 1996)
BCBL-1	Primary effusion lymphoma	KSHV	(Renne et al., 1996b)
BCP-1	Primary effusion lymphoma	KSHV	(Boshoff et al., 1998)
HBL-6	Primary effusion lymphoma	KSHV/EBV	(Gaidano et al., 1996)
JSC-1	Primary effusion lymphoma	KSHV/EBV	(Cannon et al., 2000)
JSC-1	Primary effusion lymphoma	KSHV/EBV	(Vieira and O'Hearn, 2004)
rKSHV.219		rKSHV.219	
DEL	Anablastic DLBCL		(Barbey et al., 1990)
NCI-H292	Multiple myeloma		(Gazdar et al., 1986)
Raji	Burkitt's Lymphoma	EBV	(Pulvertaft, 1964)
Daudi	Burkitt's Lymphoma	EBV	
Mutu	Burkitt's Lymphoma	EBV	

HEK 293T and derived cell lines, were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Invitrogen, Paisley, UK) with 15 % FCS and P/S. These cells were passaged three times a week and were split at either 1:4 or 1:6. HeLa and Vero derived cell lines were cultured as above, however medium was supplemented with 10 % FCS (Table 2-2).

Table 2-2 Cell lines, not of B-cell origin, used in this study

Cell line	*Description*	*Reference*
HEK 293-T	293 cells containing the temperature sensitive gene encoding SV40 T-antigen	(DuBridge et al., 1987)
rKSHV.219 HEK 293-T Clone 9/Clone5	293 cells containing the temperature sensitive gene encoding SV40 T-antigen infected with rKSHV.219	(Wilson et al., 2007)
Vero rKSHV.219	Kidney epithelial cells extracted from African Green Monkey infected with rKSHV.219	(Vieira and O'Hearn, 2004)
HeLa	Human cervical carcinoma cell line	(Masters, 2002)

Recombinant KSHV, rKSHV.219, which encodes GFP expressed from the human EF-1α promoter and DsRed expressed from by KSHV PAN promoter (Jeff Vieira, University of Washington (Vieira and O'Hearn, 2004)) infected cells were maintained with 2 µg/ml of puromycin dihydrochloride from *S. alboniger* (Sigma Aldrich, Dorset, UK). Cells were grown in a humidified incubator at 37°C with 5 % carbon dioxide (CO_2) in 21 % atmospheric oxygen (O_2) (Normoxia) or in a hypoxic chamber gassed regularly with a 5 % CO_2 and 95 % nitrogen (N_2) mix (Hypoxia) and placed within the standard incubator. Cylinders containing custom mixtures of O_2, CO_2, and N_2 were purchased from BOC (London, UK). Oxygen levels of media were measured by the FOXY-R stainless steel 1/16" OD Fibre Optic Probe (Ocean Optics) (kindly supported by Tim Arnett, University College London, (Arnett et al., 2003; Orriss et al., 2009)).

2.1.1 Freezing cells

Cells were centrifuged at 325 x g for 5 minutes and resuspended at $5x10^6$ cells/ml in cold FCS containing 10 % dimethyl sulphoxide (DMSO, Sigma, Dorset, UK). Cells were aliquoted into cryovials (Nunc, USA) and gradually cooled to –80^{o}C in an isopropanol-containing cryo-container (Nalgene, Rochester, NY, USA) overnight before being transferred to liquid nitrogen.

2.1.2 Thawing cells

Cells were removed from liquid nitrogen and thawed rapidly at 37^{o}C. The contents of the cryotube were layered onto 10 ml of RPMI - 1640 or DMEM medium, with 10 % FCS and P/S depending on cell type (see section 2.1). Cells were then pelleted at 325 x g for 5 minutes. The cell pellet was washed with 10 ml culture media. The cells were centrifuged for a further 5 minutes and

resuspended in a suitable volume of culture medium depending on the cell number, ascertained using a haemocytometer.

2.1.3 Induction of KSHV lytic replication by drug treatment

To activate protein kinase C and induce KSHV reactivation, $4x10^5$ PEL or rKSHV.219 containing cells were cultured in medium containing 20 ng/µl, 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) (Sigma Aldrich, Dorset, UK) overnight (Renne et al., 1996b). Sodium butyrate (NaBut, Sigma Aldrich, Dorset, UK) is a histone deacetylase inhibitor and leads to KSHV reactivation, cells were cultured in medium containing 2-3 mM NaBut overnight (Miller et al., 1996) (Miller et al., 1997). To induce an unfolded protein response (UPR) and the reactivation of KSHV via XBP-1s, $4x10^5$ cells were cultured in medium containing 3-4 mM dithiothreitol (DTT) overnight (Wilson et al., 2007). All drugs were removed and replaced with fresh medium the following day, and images or samples taken accordingly.

2.1.4 Deriving rKSHV.219 clonal cell populations - single colony selection

Vero cells infected with the recombinant KSHV, rKSHV.219, were treated with 3 mM NaBut (see section 2.1.3). This induced the KSHV lytic cycle as seen by an increase in DsRed-positive cells. Supernatants from these cells containing rKSHV.219 were harvested at 48 and 72 hours post NaBut treatment, passed through a 0.45 µm filter and stored at -80^{o}C in 1 ml aliquots. This virus was then used to infect HEK 293T cells by spinoculation for 1 hour at 500 x g. 48 hours post infection the medium was removed and replaced with medium supplemented with 2 µg/ml puromycin (Sigma Aldrich, Dorset, UK). The medium was removed and replaced with fresh medium supplemented with 2 µg/ml puromycin every 2-3 days until non-infected cells were no longer viable and GFP-positive single cell clones could be seen on the plate. These were then picked by suction pipetting and cultured individually in media with 2 µg/ml puromycin. The antibiotic-selected rKSHV.219 HEK 293T cell lines were then cultured normally in medium supplemented with 2 µg/ml puromycin and analysed for spontaneous lytic replication (DsRed expression) and reactivation in response to various stimuli.

2.2 Flow cytometry

Ten thousand events were collected using a FACScan, FACsArray or LSR flow-cytometers with Cellquest software (Becton Dickinson, Oxford, UK). Data was analysed using Windows Multiple Document Interface Flow Cytometry Application (WinMDI, J. Trotter, http://facs.scripps.edu).

2.2.1 Preparation of live cells for flow cytometry

48 hours post infection cells were washed once with PBS, and incubated for 1 minute with trypsin-EDTA (GIBCO, Invitrogen, Paisley, UK) at room temperature. The trypsin was then quenched with DMEM supplemented with 10 % FCS and P/S and transferred to sealed FACS tubes (Falcon). The cells were pelleted, resuspended in PBS plus 3.7 % paraformaldehyde (TAAB) and kept on ice prior to analysis.

2.3 Microscopy

2.3.1 Confocal microscopy

Images of GFP and DsRed fluorescence were gathered by laser scanning confocal microscopy (Zeiss Axiovert 100 TV) using Lasersharp 2000 (Bio-Rad). Cells were excited at 488 nm to image enhanced GFP fluorescence and separately excited at 568 nm to image DsRed expression with negligible GFP excitation and the equivalent intensity for each sample. Imaging of live cells was achieved through replacing the growth medium with PBS. No image enhancement was used.

2.3.2 Light microscopy

Cell were visualised using a Nikon Eclipse TS100 and images of GFP and RFP fluorescence were gathered using a digital camera Nikon E950. Imaging of live cells was achieved through growth medium.

2.4 General Molecular biology Techniques

2.4.1 Preparation of competent bacteria

XL-1 Blue *Escherichia coli* (Stratagene, Leicester, UK) stored in the form of a glycerol stock were streaked on a 1.5 % Luria-Bertani agar plate, (LB, 10 g

trypton, 5 g yeast extract and 5 g sodium chloride plus 1.5 % agar-agar per litre of medium) containing 10 µg/ml tetracycline and incubated at 37^{o}C for 16 hours. A single colony was used to inoculate 5 ml of LB-broth, supplemented with 50 µg/ml tetracycline, and shaken at 37^{o}C for 16 hours. This was added to 500 ml LB-broth (as above composition without added agar) and shaken at 37^{o}C until the absorbance at 550 nm was 0.45-0.55 (around 3 hours). The culture was then put on ice to cool for 10 minutes. The bacteria were pelleted at 3000 x g for 10 minutes at 4^{o}C and resuspended in 15 ml of 100 mM calcium chloride (4^{o}C). After incubation on ice for 30 minutes, the bacteria were centrifuged again and then resuspended in 2.5 ml of 100 mM calcium chloride containing 15 % glycerol by volume (4^{o}C). The bacterial suspension was frozen on dry ice in 400 µl aliquots and stored at -80 ^{o}C.

2.4.2 Introduction of plasmid DNA into *E. coli* by heat shock

1-200 ng of plasmid was mixed with 50 µl of competent XL-1 Blue *Escherichia coli* (Stratagene, Leicester, UK) that have been thawed on ice and incubated on ice for 30 minutes. The cells were then heat shocked for 45 seconds at 42^{o}C and immediately incubated on ice for 2 minutes. 450 µl of LB-broth was added to the heat shocked bacteria and incubated at 37^{o}C for 1 hour with shaking at 250 rpm. 100 µl of the culture was then plated on LB-agar plates containing 50 µg/ml ampicillin and incubated upside down overnight at 37^{o}C.

2.4.3 Plasmid DNA midi-preps

Midi preps of plasmid DNA were produced from 100 ml overnight liquid cultures of transformed XL-1 Blue *Escherichia coli* using the Plasmid Midi Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

2.4.4 Plasmid DNA mini-preps

Mini preps of plasmid DNA were produced from 5 ml overnight liquid cultures of XL-1 Blue *Escherichia coli* using the QIAprep Spin Miniprep Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

2.4.5 Molecular Cloning

KOD Hot start Polymerase with proof-reading activity was purchased from Merck Chemicals Ltd., Nottingham, UK. Restriction endonucleases were

purchased from Promega, Southampton, UK or New England Biolabs, Hitchin, UK. Calf intestinal alkaline phosphatase and T4 DNA ligase were purchased from Promega, Southampton, UK. All were used in accordance with the manufacturer's specifications.

2.4.5.1 Cloning using the pGEM-T-Easy vector system (Promega, Southampton, UK)

The desired region was PCR-amplified using 1 unit of KOD Hot start polymerase (Merck Chemicals Ltd., Nottingham, UK) in a 50 µl reaction volume buffered with KOD Hot start polymerase 10 x buffer (Merck Chemicals Ltd., Nottingham, UK). PCR conditions involved 10-100 ng of template DNA and 10 picomoles of each oligonucleotide primer. In addition, the reaction was supplemented with a final concentration of 0.2 mM dNTPs (Merck Chemicals Ltd., Nottingham, UK) and 1.5 mM Mg++ (Merck Chemicals Ltd., Nottingham, UK). Cycling conditions were used as manufacturer's instructions.

The desired PCR product is then purified using a Qiaquick gel extraction kit (Qiagen, Crawley, UK). Briefly 500 µl of QC buffer was added directly to PCR reaction mix, 10 µl of isopropanol was added and mixed well before all 650 µl were applied to the column. Manufacturer's instructions for gel extraction were then followed and the PCR product was eluted in 32 µl. In order to allow cloning via the pGEM-T-Easy vector system the PCR product must have overhanging dATP ends. This was achieved via a 10 minute incubation with 1.5 units GoTaq polymerase and 1.6 µl of 5 nM dATPs in 8 µl of GoTaq buffer with $MgCl_2$ and 28 µl of PCR product at 72°C.

The correct molecular weight product was then separated by electrophoresis and visualised using, 10 mg/ml ethidium bromide (Sigma Aldrich, Dorset, UK) stained, TAE buffered agarose gel (Sigma Aldrich, Dorset, UK) with a percentage between 1-3 % depending on size of expected product. The product was subsequently excised and extracted using the MinElute gel extraction system (Qiagen, Crawley, UK) in accordance with the manufacturer's instructions.

The following ligation reaction was then used using the pGEM-T easy vector system (Promega, Southampton, UK). Negative controls where PCR-amplified DNA is substituted with H_2O were ligated in parallel.

5 µl 2X ligation buffer
1 µl T4 DNA ligase
0.5 µl pGEM-T-Easy linear DNA
3.5 µl MinElute extracted PCR product

Ligation reactions were transformed as described in section 2.4.2 and positive colonies were identified by blue-white-screening, PCR-screening or restriction enzyme digest as desired.

2.4.5.2 Subcloning

Primers were designed to contain restriction endonuclease sites to subclone the gene of interest from pGEM-T easy vector into appropriate expression construct or lentiviral vector (Table 2-3) (see section 2.4.5.3). Vectors were digested overnight with appropriate restriction endonucleases as per manufacturer's instructions and then treated with calf intestinal alkaline phosphatase (Promega, Southampton, UK) before being purified using the MinElute gel extraction system (Qiagen, Crawley, UK) in accordance with the manufacturer's instructions. The pGEM-T vector containing gene of interest was checked by digestion overnight with appropriate restriction endonucleases. The insert, (i.e. gene of interest) is purified using the MinElute gel extraction system (Qiagen, Crawley, UK) and cloned into the appropriate expression vector (see section 2.4.5.3). Ligations were carried out using the following ligation reaction. Negative controls are where PCR-amplified DNA is substituted with H_2O were ligated in parallel.

5 µl 2X ligation buffer (Promega, Southampton, UK)
1 µl T4 DNA ligase (Promega, Southampton, UK)
1 µl Linear alkaline phosphatase treated vector
3 µl MinElute extracted PCR product

Ligation reactions were transformed as described in section 2.4.2 and positive colonies were identified by PCR-screening or restriction enzyme digest as desired.

Table 2-3 DNA primers for cloning (Sigma Aldrich, Dorset, UK)

Oligo Number	*Name*	*Sequence*
1	HIF-1α fwd	ggatccgagggcgccggcggcgcgaac
2	HIF-1α rev	gcggccgcgttaacttgatccaaagctc
3	RTA prom fwd	gcgctagcaattggaagcattctctct
4	RTA prom rev	gcagatctggaccgccgaagcttcttacc
5	RTA DN fwd	gcagatctatgaaagaatgttccaag
6	RTA DN rev	gcgcggccgccaccttcctcttcttcttgg gtagactctgatctacgtccag

2.4.5.3 Lentivirus vector maps

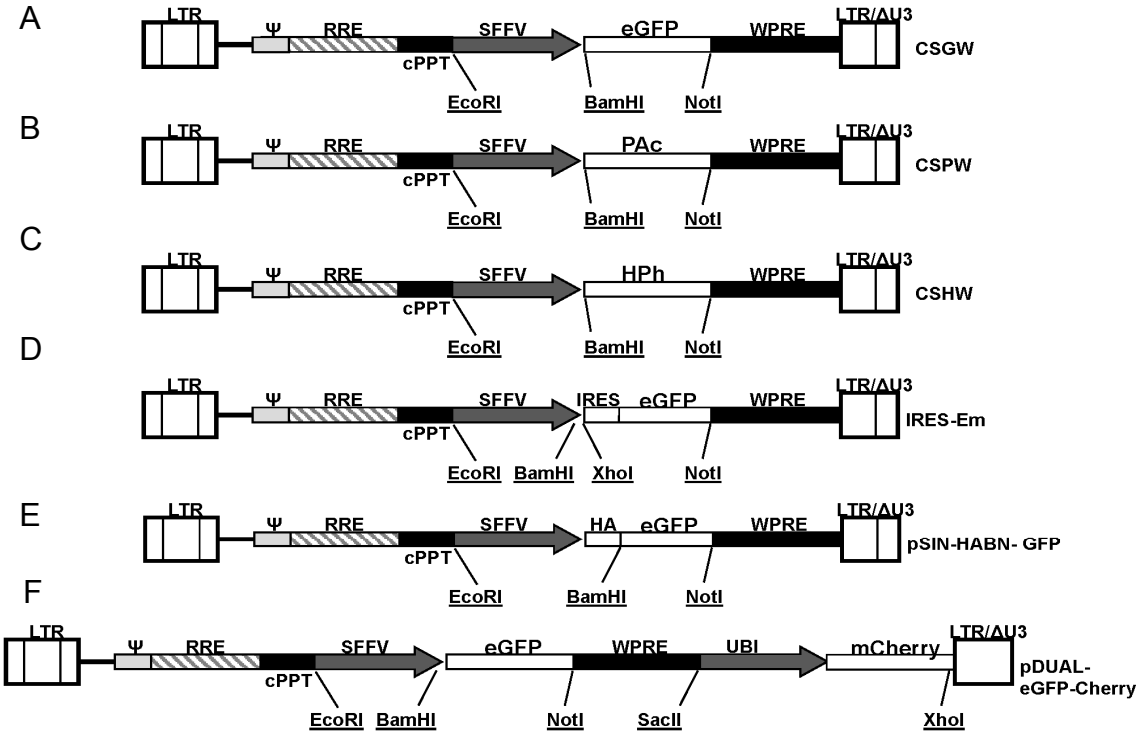

Figure 2-1 Lentivirus vector maps (see table in Appendix)
Plasmids A is described in (Demaison et al., 2002) and plasmids B-F are modified versions of A. All plasmids contain integrative long terminal repeat regions (LTRs), a packaging signal (Ψ), a Rev response element (RRE) to promote mRNA nuclear export, a central polypurine tract (cPPT) for nuclear import and increased integration, a Spleen focus forming virus (SFFV) promoter and aa wood chuck hepatitus virus posttranscriptional regulatory element (WPRE) to promote mRNA maturation. Plasmid A was obtained from the laboratory of Professor Mary Collins and encodes eGFP under the SFFV promoter which can be substituted. Plasmids B and C encode puromycin resistance gene (PAc) and the hygromycin resistance gene (HPh) respectively. Plasmid D as internal ribosome entry site (IRES) to allow GFP to be expressed as a marker. Plasmid E has been modified to include an HA tag that is added to any cloned protein and finally plasmid F contains an addition Ubiquitin (Ub) promoter to allow dual expression.

2.4.5.4 Hypoxia Inducible Factor-1 α (HIF-1α)

Lentiviral vector-genome expressing HIF-1α was generated by amplifying HIF-1α using primers 1 and 2 (Table 2-3) and pCDNA3 HIF-1α (a kind gift from Peter Ratcliffe) as a template. The resulting 2.4 kb amplicon was cloned into pGEM-T-Easy vector (see section 2.4.5.1) (Promega, Southampton, UK) and sequence verified. The BamHI/NotI restricted HIF-1α insert was then subcloned into a modified version of the lentivirus vector pHR-SIN-CSGW (pCSGW), pSIN-HABN, kindly provided by Dr Edward Tsao, see section 2.4.5.3 allowing the expression of N-terminal HA-tagged HIF-1α (pSIN-HABN- HIF-1α/pSIN-HA-HIF-1α) (see table in Appendix).

2.4.5.5 Luciferase reporter plasmids

A wild-type and a mutant XBP-1 response element containing 4099 bp region of the ORF50 promoter, first exon, intron and the first 7 codons of the second exon was PCR amplified using primers 3 and 4 (Table 2-3) and 100 ng of template p50Redi previously described (Wilson et al., 2007) or a mutated version of this promoter pMUT50redi respectively. pMUT50redi was created by Sam Wilson using the QuickChange mutagenesis system (Stratagene, Leicester, UK) and the primers forward (5'-GCT TTT CAG GAG AGT TAG GTC GAC ACT GAG GAT GTG GAC AAG CTT CTG C-3') and reverse (5'- GCA GAA GCT TGT CCA CAT CCT CAG TGT CGA CCT AAC TCT CCT GAA AAG C -3') following manufacturer's instructions (Dalton-Griffin et al., 2009). The PCR-products were then cloned into pGEM-T-Easy (see section 2.4.5.1). The 4099 bp wild-type or mutant fragments were then digested with NheI and BglII and sub-cloned into pGL2-Basic vector (Promega, Southampton, UK), see section 2.4.5.2, to produce the reporter constructs p50LUC and p50MUTLUC respectively (see Figure 3-8) (see table in Appendix).

2.4.5.6 RTA dominant negative (DNRTA)

Lentiviral vector-genome expressing the C-terminal truncated, dominant negative form of RTA (Lukac et al., 1999) was generated by amplifying the first 1590 bp of RTA using primers 5 and 6 (Table 2-3) and pSIN-BNHA-kRTA kindly provided by Dr Edward Tsao as a template. The resulting 1.5 kb amplicon was cloned into pGEM-T-Easy vector (see section 2.4.5.1) (Promega, Southampton, UK) and sequence verified. The BglII/NotI restricted DNRTA insert was then

subcloned into a modified version of the pSIN-BNHA, kindly provided by Dr Edward Tsao, (see section 2.4.5.3) allowing the expression of C-terminal HA-tagged DNRTA (pSIN-BNHA-DNRTA/pSIN-DNRTA). The BglII/NotI restricted DNRTA fragment was also subcloned into the dual promoter lentivirus vector (pDUAL-MEK-Cherry - provided by David Escors see section 2.4.5.3), to give pDUAL-DNRTA-Cherry(see table in Appendix). This vector expresses the gene of interest under the SFFV promoter and mCherry under the control of the ubiquitin promoter.

2.4.6 Generation of lentiviral vectors encoding shRNAs

The lentiviral vector expressing the short hairpin RNA (shRNA)-RTA, XBP-1 and -GFP were produced previously (Wilson et al., 2007). The lentiviral vectors expressing shRNA to HIF-1α or Luciferase were generated using same methodology. Briefly, the forward primer 7 and reverse primer 8, 9 or 10 (see Table 2-4) were used to PCR amplify a shRNA expression cassette using the plasmid pGemU61Linker previously described (Wilson et al., 2007). This amplicon was cloned into the pGEM-T-Easy vector (see section 2.4.5.1), sequence verified, and subcloned (see section 2.4.5.2) via EcoRI restriction into antibiotic resistance modified versions of pCSGW namely the puromycin acetylase (PAc) encoding pHR-SIN-CSPW (pCSPW) or the hygromycin phosphotransferase (HPh) encoding pHR-SIN-CSHW (pCSHW) (see table in Appendix). shRNAs were sequenced using oligonucleotide 7 (Table 2-4) in their lentiviral vector context prior to use.

Table 2-4 Oligonucleotides used to construct shRNA expression constructs (Sigma Aldrich, Dorset, UK)

Oligo Number	***Name***	***Sequence***
7	U6 fwd	gggctgcagaaggtcgggcaggaagagggcctatttccc
8	HIF-1 α 1545	cgaaaaagatgaccagcaacttgaggaagtCGAAacctccccaag ctactggtcatcggtgtttcgtcctttccacaagatatataaag
9	HIF-1α 1589	cgaaaaagaattcaagttggaattggtagaCGAAtccaccaactc caacctaaattcggtgtttcgtcctttccacaagatatataaag
10	Luciferase 1343	cgaaaaaggtggctcccgctgaattggaatCGAAatcccaatcca gcgagagccaccggtgtttcgtcctttccacaagatatataaag

2.4.7 DNA sequencing

Cloning was confirmed by sequencing carried out either by Cogenics (Lark Technologies Incorporated, Essex, UK) or the University College London sequencing service. Results were then analysed using the program DNA dynamo.

2.4.8 Expression plasmids

The plasmid pXBPsIG was a generous gift from C. Tsantoulas, who generated pXBPsIG under the supervision of S. Wilson during an MSc project in the Kellam laboratory. The pXBPsIG construct encodes XBP-1s tagged with a c-terminal His-tag cloned in the IRES-Em vector. XBP-1s tagged with a c-terminal His-tag cloned in pCDNA3 (GIBCO, Invitrogen, Paisley, UK) was also supplied by S. Wilson. Another XBP-1s-expressing construct was generated by Edward Tsao, the Dual promoter lentivirus backbone pDUAL-XBP-1s-Cherry (provided by David Escors) (see table in Appendix).

Positive control for HIF-1α expression, (pCDNA3- HIF-1α) was kindly donated by Peter Ratcliffe, University of Oxford (Raval et al., 2005)). A plasmid expressing viral RTA from the HCMV promoter (pCMVRTA) was supplied generously by Adrian Whitehouse, University of Leeds (Wilson et al., 2007). Finally, RTA-expressing pSIN-BNHA-kRTA and a GFP-expressing, control plasmid pSIN-BNHA-GFP/pSIN-GFP were supplied by Edward Tsao(see table in Appendix).

2.4.9 Reporter plasmids

The luciferase reporter plasmid pRpluc1-3087 contains a 3-kb sequence upstream of the RTA transcriptional start that drives the expression of firefly luciferase. Truncated RTA promoter pRpluc 1115-1327 contains the hypoxia response element 2, (HRE2) only. Truncated RTA promoter pRpluc1-550 contains hypoxia response element 4, (HRE4) also identified as XBP-1 response element (XRE) only. All luciferase plasmids outlined above were kind gifts from Erle Robertson, University of Pennsylvania. (Cai et al., 2006a) (see Figure 4-10) (see table in Appendix).

2.5 Transient transfection

2.5.1 Transient transfection of HEK 293-T cells

$2x10^5$ HEK 293-T cells were seeded per well of a six-well plate. The following day the culture medium was removed and replaced with fresh medium. The transfection was carried out using FuGENE-6 transfection reagent (Roche Applied Science, Burgess Hill, UK) as per the manufacturer's instructions. Briefly, transfection mixes including 6 µl of FuGENE-6, 100 µl of Opti-MEM (GIBCO, Invitrogen, Paisley, UK) and varying amounts of DNA were incubated at room temperature for 30 minutes and then added drop wise to the cells. The amounts of Opti-MEM and FuGENE-6 were adjusted according to the well size and cell number by ratio. The medium containing the transfection mixture was removed the day after transfection and replaced with fresh medium. 48 hours post-transfection live cells were analysed by flow cytometry see section 2.2, RT PCR see section 2.7, western blot see section 2.8 or luciferase assay 2.9.

2.6 Lentivirus production, transduction and titration

2.6.1 Transient transfection of HEK 293-T cells for the production of lentivirus.

Lentiviral vectors were produced as described in (Besnier et al., 2002). HEK 293-T cells were seeded so that 10 cm dishes were confluent on the day of transfection. 10 µl of FuGENE-6 (Roche Applied Science, Burgess Hill, UK) was added to 200 µl of Opti-MEM (Invitrogen, UK). 1 µg of p8.91, 1 µg of pMDG (both generous gifts from Didier Trono) and 1.5 µg of vector-genome encoding plasmid DNA were made up to 15 µl in H_2O and added to the FuGENE-6 and Opti-MEM mixture. The transfection mixture was incubated at room temperature for 30 minutes before being added drop wise to the confluent HEK 293-T cells in 8 ml DMEM medium with 10 % FCS and P/S.

The HEK 293-T cells and the transfection reaction were incubated overnight at 37^oC and 10 % CO_2. The lentiviral vector containing supernatants were harvested at 48, 72 and 96 hours post transfection, passed through a 0.45 µm filter and stored at -80 ^oC in 1 ml aliquots.

Virus was concentrated from the pooled supernatant collections using polyethylene glycol molecular weight 8000 (PEG – Sigma, Dorset UK)) treatment. 1/10th the volume of sterile 50 % PEG solution and 1/33rd the volume of 5 M sodium chloride (Sigma Aldrich, Dorset, UK) was added to the filtered supernatants, mixed well by inversion and stored at 4°C overnight (these are stable at 4°C at least a week). The virus collections were centrifuged at 2000 x g at 4°C for 10 minutes the supernatant was then discarded carefully to avoid disturbing the pellet. The virus pellet was finally resuspended in media at a low volume by pipetting up and down several times. Concentrated virus was then aliquoted and stored at -80°C.

2.6.2 Lentiviral vector titration of infectious units by GFP or RFP expression

1x10^5 HEK 293-T cells were seeded per well of a 24-well plate and cultured overnight at 37^{o}C and 10 % CO_2 in 1ml of HEK 293-T medium (DMEM containing 10 % FCS, P/S). 1:3 serial dilutions of supernatant containing lentivirus were made using HEK 293-T medium. 100 µl of the diluted lentivirus containing supernatants and 100 µl of undiluted supernatant supplemented with 15 µg/ml polybrene (Sigma Aldrich, Dorset, UK) were added to HEK 293-T cells in 0.9 ml of fresh medium. Cells were spinoculated for 1 hour at 500 x g. Cells were then incubated overnight at 37^{o}C and 10 % CO_2. The cells were washed once with HEK 293-T medium and incubated for a further 36 hours.

After incubation the HEK 293-T cells were trypsinised and the percentage of GFP- or RFP-expressing cells determined by flow cytometry (see section 2.2) relative to non-infected cells. The percentage of GFP- or RFP-expressing cells were converted to infectious units by assuming a total of 2x10^5 cells at the time of infection. Infectious units were plotted against µl of supernatant. The absolute titre of the lentiviral vector was determined by extrapolating the infectious units per ml (IU/ml) using the straight-line equation of the above plot.

2.6.3 Lentiviral vector titration of infectious units by antibiotic selection and colony counting

1x10^5 HEK 293-T cells were seeded per well of a 24-well plate and cultured overnight at 37^{o}C and 10 % CO_2 in 1 ml of HEK 293-T medium (DMEM

containing 10 % FCS and P/S). 1:10 serial dilution of supernatant containing lentivirus were made so as 10 µl, 1 µl, 0.1 µl, 0.01 µl and 0.001 µl of lentivirus containing supernatants were mixed with HEK 293-T medium to a total volume of 100 µl. The diluted lentiviral-vector containing supernatants were supplemented with 15 µg/ml polybrene (Sigma Aldrich, Dorset, UK) and added to HEK 293-T cells. Cells were spinoculated for 1 hour at 500 x g and then incubated overnight at 37^{o}C and 10 % CO_2. The cells were then trypsinised and cultured in 5 cm dishes containing 5 ml of HEK 293-T medium containing the appropriate amount of antibiotic depending on the resistance encoded by the lentivirus (see section 2.6.6). 3 days after infection the plates were washed and placed in fresh HEK 293-T medium containing antibiotic. 14 days after infection visible antibiotic resistant colonies were counted and the infectious units extrapolated from the dilution containing approximately 10 colonies.

2.6.4 Lentiviral transduction of suspension cells

0.5x10^5 cells per well of a 24-well plate in 1 ml of the required amount of, lentivirus containing, HEK 293-T supernatant and RPMI culture medium supplemented with 15 µg/ml polybrene (Sigma Aldrich, Dorset, UK). Cells were spinoculated for 1 hour at 500 x g. The cells were then incubated for 6 hours or overnight at 37^{o}C and 5 % CO_2. The cells were then pelleted and resuspended in fresh RPMI medium.

2.6.5 Lentiviral transduction of adherent cells

1 x10^5 cells were plated per well of a 24-well plate. The following day the medium was removed and replaced with 1 ml of the required amount of, lentivirus containing, HEK 293-T supernatant supplemented with 15 µg/ml polybrene (Sigma Aldrich, Dorset, UK).and culture medium. Cells were spinoculated for 1 hour at 500 x g. The cells were then incubated for 6 hours or overnight at 37^{o}C. The lentiviral vector containing, HEK 293-T supernatant and culture medium was then removed and replaced with fresh medium.

2.6.6 Antibiotic selection following lentiviral vector transduction

48 hours post infection (transduction see sections 2.6.4 and 2.6.5) the medium was removed and replaced with medium supplemented with 2 µg/ml puromycin dihydrochloride from *S. alboniger* (Sigma Aldrich, Dorset, UK) or 750 µg/ml

hygromycin B from *S. hygroscopicus* (GIBCO, Invitrogen, Paisley, UK) depending on the encoded resistance. The medium was removed and replaced with fresh medium supplemented with appropriate antibiotic every 2-3 days until non-transduced cells, in parallel cultures, were no longer viable. The antibiotic-selected cells were then cultured normally in medium supplemented with respective antibiotic.

2.7 RT PCR

2.7.1 RNA extraction and DNase treatment

Total RNA was purified from 8-10x10^5 cells resuspended in 1 ml of Trizol (GIBCO, Invitrogen, Paisley, UK). After an initial chloroform extraction step RNA was isolated using an RNeasy mini extraction kit (Qiagen, Crawley, UK) including an on-column DNase (Promega, Southampton, UK) digestion following the manufacturer's instructions. RNA was quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE).

2.7.2 cDNA synthesis

Reverse transcription reactions were carried out using Omniscript Reverse Transcriptase (Qiagen, Crawley, UK), in accordance with the manufacturer's instructions and starting with 1-2 µg of total RNA.

2.7.3 PCR to detect messenger RNA

The desired region was PCR-amplified from the oligonucleotide dT (GIBCO, Invitrogen, Paisley, UK)-primed cDNA using 5 units of GoTaq polymerase (Promega, Southampton, UK) in a 50 µl reaction volume buffered with 5x GoTaq polymerase flexi buffer (Promega, Southampton, UK). PCR conditions involved 20 picomoles of each oligonucleotide primer. In addition, the reaction was supplemented with a final concentration of 0.2 mM dNTPs (Promega, Southampton, UK) and between 0.5 and 2 mM $MgCl_2$ (Promega, Southampton, UK).

Primers were designed to amplify a 300 bp region towards the 3' end of the gene of interest. β-actin and GAPDH were detected using primers a and b or c and d respectively (Table 2-5). The PCR program used was as follows: hotstart

of 95°C for 2 minutes a PCR cycle consisting of 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute this was repeated 28 times. RTA was amplified from KSHV containing cells using primers i and j (Table 2-5) and the same cycling conditions as for β-actin above however with 35 cycles. ORF29a/b was amplified using the primers g and h (Table 2-5). The PCR reaction contained 1.8 mM $MgCl_2$ and the same PCR cycle as above except with an annealing temperature 65°C and 35 cycles. All PCR programs finish with a final 5 minutes extension at 72°C.

The correct molecular weight product was separated and visualised using, 10 mg/ml ethidium bromide (Sigma Aldrich, Dorset, UK) stained, agarose gel electrophoresis as before (see section 2.4.5.1).

2.7.3.1 XBP1 PCR, *Pst* I digestion and resolution

PCR amplification across the XBP-1 atypical splice junction was achieved using oligonucleotides e and f (Table 2-5). The desired region was PCR-amplified as above section 2.7.3., however with an annealing temperature of 58^{o}C and with 35 cycles. Following PCR amplification the 50 µl reaction was halved into 25 µl. Then 1 µl of H_2O was added to one half while 20 units of *Pst* I was added to the other half of the reaction and incubated overnight at 37^{o}C. The restricted and non-restricted PCR products were resolved using agarose gel electrophoresis. The best separation was achieved using 3 % TBE buffered agarose gels, pre-cooled to 4 ^{o}C. The gels were resolved at 5 volts/cm in room temperature TBE buffer diluted 1:1 with H_2O.

Table 2-5 DNA primers for mRNA detection (Sigma Aldrich, Dorset, UK)

Oligo	*Name*	*Sequence*
a	Beta Actin fwd	ctgtggcatccacgaaacta
b	Beta Actin rev	acatctgctggaaggtggac
c	GAPDH fwd	atggggaaggtgaaggtcgg
d	GAPDH rev	tggtgaagacgccagtggac
e	XBP-1short fwd	ccttgtagttgagaaccagg
f	XBP-1short rev	cagaatgcccaacaggatatc
g	ORF29a/b fwd	gcacgtagccaactccgtg
h	ORF29a/b rev	gcaggaaactcgtggagcg
i	RTA fwd	aatgcgttacgttgttgcag
j	RTA rev	tcccaaagaggtaccaggtg

2.8 Immunoblotting

Approximately $8x10^5$ cells were lysed directly with 100 µl sample buffer (0.2 M Tris-HCl pH 6.8, 5.2 % sodium dodecyl sulphate (SDS), 20 % glycerol, 0.1 % bromophenol blue and 40 mg/ml DTT on day of use). Samples were then sonicated and boiled then centrifuged at 15,500 x g for 1 minute. Samples were aliquoted into 20 µl (load volume) and stored at -20°C. Protein concentration was normalised by cell number and by blotting for a housekeeping protein. Proteins were resolved by 10 % SDS poly-acrylamide gels using Bio-Rad (Hemel Hempstead, UK) apparatus and SDS PAGE running buffer (10x in 5 litres: 50 g SDS, 150 g Tris, 720 g glycine. Separated proteins were then transferred using a semi-dry transfer blotter (Amersham Biosciences) and semiphor transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol) to a polyvinylidene difluoride membrane (PVDF, GE Healthcare, Little Chalfont, UK). Blotting conditions were 0.8 mA/cm^2 for 1 hour.

After blocking for 1 hr with 5 % non-fat dry milk in TBS (10x in 5 litres: 120.5 g Tris-base, 400 g NaCl, pH 7.6) containing 0.1 % TWEEN 20 (Sigma Aldrich, Dorset, UK) (TBST), the membrane was probed overnight at 4 °C with primary antibodies against HIF-1α (monoclonal BD Transduction Laboratories, San Jose, CA 1/200), RTA (polyclonal, Don Ganem, University of California and San Francisco (Lukac et al., 1998)1/20,000), BZLF-1: BZ-1 (Imperial College London, Dr. Paul Farrell (Bryant and Farrell, 2002)1/10,000), HIS (GE Healthcare Little Chalfont, UK 1/1000) or XBP-1(s) (monoclonal, Giovanna Roncador (Maestre et al., 2009) 1/100) in 1 % non-fat dry milk. Blots were washed 5 times with TBST at intervals of 5 minutes each before 1 hr incubation with the appropriate horseradish peroxidase (HRP) conjugated secondary antibodies (GE Healthcare, Little Chalfont, UK) in 1 % non-fat dry milk in TBST at room temperature. The primary antibodies anti-HA (HRP-conjugated 1/3000) and β-actin (ABcam 1/40,000) as well as corresponding secondary antibody (1/2000) as required were incubated for 1 hour at room temperature respectively in TBST. All blots were washed 5 times with TBST at intervals of 5 minutes each before developing. HRP-conjugated antibody was detected using ECL western blotting detection reagent (GE Healthcare, Little Chalfont,

UK). Chemiluminescence was measured by autoradiography using Hyperfilm ECL (GE Healthcare, Little Chalfont, UK).

2.9 Luciferase promoter assays

2.9.1.1 Bright Glo protocol

HEK 293T cells were plated at a density of $2x10^4$ in 96-well plates, the next day cells were transfected using 20 ng of the appropriate ORF50 reporter plasmids, section 2.4.9 (a kind gift from Erle Robertson, University of Pennsylvania, (Cai et al., 2006a) in combination with molar equivalent transcription factor expressing plasmid DNA, see section 2.5.1. After 48 hours, quantification of relative light units (RLU) was determined using the luciferase Bright Glo reagent in accordance with the manufacturer's instructions (Promega, Southampton, UK) and a GloMax 96-microplate luminometer with single injector (Promega, Southampton, UK). All assays were performed in triplicate. Transfection efficiency was monitored by parallel transfection with a fluorescent protein and flow cytometry. The standard error of the mean for each triplicate represents the error for each experiment.

2.9.1.2 Dual luciferase assay protocol

As above with a few exceptions, reporter plasmids used were described in section 2.4.5.5, in addition 2 ng of *Renilla* Luciferase expression plasmid (phRL-null) plasmid (a kind gift from Professor Gary Stein, University of Massachusetts) was transfected into every well to act as transfection control and used to normalise firefly luciferase data. After 48 hours, cells were harvested and lysates were measured for luciferase activity with a dual-luciferase reporter assay kit (Promega, Southampton, UK) in accordance with manufacturer's instructions. Raw values obtained from each experiment, performed in triplicate, were normalized to the *Renilla* value for each replicate. The standard error of the mean for each triplicate represents the error for each experiment.

2.10 Virion DNA extraction and TaqMan PCR

180 µl from 1 ml of supernatant containing approx $1\text{-}2x10^5$ PEL cells in culture under normoxia or hypoxia were treated with DNase (Promega, Southampton,

UK), followed by the addition of 10 µg of salmon sperm DNA (ssDNA) as carrier. Virion DNA was extracted using the QIAamp DNA extraction kit (Qiagen, Crawley, UK). The amount of recovered DNA was used to normalise the DNA input of different samples for the calculation of viral load. KSHV ORF37 TaqMan PCR was performed as described previously with modification (Stamey et al., 2001). Each 25 µl PCR contained 1x Platinum Quantitative PCR SuperMix-Uracil DNA Glycosylase (UDG) with ROX (GIBCO, Invitrogen, Paisley, UK), 300 nM each ORF37 primer, 150 nM ORF37 probe (Sigma Aldrich, Dorset, UK), and 5 µl sample DNA. Following 2 minutes incubation at 50°C for activation of the UDG, the sample is denatured for 3 minutes at 95°C. Fifty cycles of standard quantitative PCR were performed consisting of 95°C for 15 seconds and 60°C for 50 seconds. Amplification was carried out with an ABI Prism 7000 sequence detection system (Applied Biosystems). Each PCR run also contained a standard dilution curve of ORF37 and no template negative controls. All samples were run in duplicate reactions.

2.11 Whole Human Genome Oligo Microarray analysis

2.11.1 RNA extraction

RNA was extracted as in section 2.7.1. RNA integrity was measured using RNA 6000 Series II Nano Kit and 2100 Bioanalyzer Series C (Agilent Technologies, West Lothian, UK), according to manufacturer's instructions. RNA samples used for analysis had an RNA integrity number (RIN number) of >9.0; indicating high-quality RNA with minimal degradation products. All chips were analyzed using 2100 Expert software version B.02.04 (Agilent Technologies, West Lothian, UK).

2.11.2 Labelling, hybridisation and analysis

Agilent Whole Human Genome Oligo Microarrays (G4112A) which contains 44,000 60-mer oligonucleotide probes representing 41,000 unique genes and transcripts were used for the gene expression experiments. Probe labelling and hybridization were carried out following the manufacturer's specified protocols (Two-colour Microarray-Based Gene Expression Analysis Protocol, Agilent Technologies, West Lothian, UK). Briefly, amplification and labelling of 500 ng of total RNA and was performed using Cy5 for sample RNA and Cy3 for the

universal human reference RNA (Stratagene, Leicester, UK). Hybridization was performed for 17 hours at 65°C and arrays were scanned on an Agilent DNA microarray scanner (Agilent Technologies, West Lothian, UK). Images were analysed and the data was extracted, background subtracted and normalised using the standard procedures of Agilent Feature Extraction software (v9.5.3.1) (Agilent Technologies, West Lothian, UK). Intensities for each sample relative to the reference were obtained from the feature extraction file by background subtraction and scale normalisation using R and BioConductor (Gentleman et al., 2004). For detailed analysis methods, performed by Dr Dan Frampton; see results sections 3.2.6 and 5.2.5.

2.12 Statistics

Performed by Dr Dan Frampton

3 Establishing a model for lytic reactivation - rKSHV.219 HEK 293T cells

3.1 Introduction

KSHV establishes life-long persistent latent infection of its host; however, reactivation and lytic replication is necessary for transmission and dissemination of the virus as well as tumourigenesis (Whitby et al., 1995) (Staskus et al., 1997) (Song et al., 2004) (Grundhoff and Ganem, 2004). This is highlighted by the reported reduced risk of developing Kaposi Sarcoma (KS) after treatment with ganciclovir; an anti-herpetic that is only effective against lytic virus (Martin et al., 1999). Lytic gene products have also been linked to the pathology seen in KSHV associated diseases, for example ORF74 which encodes a viral G-protein coupled receptor and induces production of the cytokine, VEGF, leading to cell proliferation and angiogenesis (Bais et al., 1998) (Liu et al., 2001) (Jenner and Boshoff, 2002) (see section 1.1.12).

The reactivation of KSHV from latency can be induced *in vitro* by various factors including the chemical inducer, 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) (Renne et al., 1996b) (Yu et al., 1999) (see section 1.1.14) However, such predominantly non-physiological inducers provide limited insight into the cellular cues that induce KSHV lytic replication in KS, PEL and MCD. The viral protein regulator of transcription activation (RTA), encoded by open reading frame 50 (ORF50), is both necessary (Xu et al., 2005) (Lukac et al., 1999) and sufficient (Gradoville et al., 2000) for inducing KSHV lytic replication. RTA can also auto-activate the ORF50 promoter leading to a substantial amplification of any stimulus that induces RTA expression (Deng et al., 2000). Despite this knowledge, what activates the switch from latency to lytic replication is still poorly understood. Therefore, determining the physiological triggers that induce RTA expression is critical for understanding the control of KSHV reactivation in asymptomatic infection and disease.

In this chapter we characterise a system for monitoring KSHV reactivation and employ this method to investigate a physiological trigger of lytic replication.

Recombinant KSHV systems, that contain a GFP expression cassette as a marker of infection, have been engineered partly to overcome the problem of KSHV propagation and dilution *in vitro* (Bechtel et al., 2003) (Zhou et al., 2002) (Vieira et al., 2001). Recent advances have added a DsRed expression cassette under the control of the KSHV lytic Polyadenylated nuclear RNA (PAN) promoter to produce the recombinant virus, rKSHV.219 (Figure 3-1 B) (Vieira and O'Hearn, 2004). The viral PAN promoter is transactivated specifically by RTA and therefore DsRed expression directly infers lytic replication. In the Vieira study the rKSHV.219 virus established latent infection and could be induced to reactivate in all cell lines tested (Vieira and O'Hearn, 2004). This system, therefore, is a simple way of monitoring KSHV reactivation in response to stimuli. The rKSHV.219 Vero and rKSHV.219 JSC-1 cell lines were generously supplied by the laboratory of Jeff Vieira.

HEK 293T cells were infected with rKSHV.219 and characterised. Using the cell lines generated we further establish the role of the transcription factor, X-box binding protein-1 (XBP-1), as an inducer of the KSHV lytic cycle (Wilson et al., 2007) (Sun and Thorley-Lawson, 2007) (Yu et al., 2007a). XBP-1, is a basic leucine zipper transcription factor essential for the induction of the unfolded protein response (UPR), which is triggered by a variety of different cellular stresses, including the ER-stress induced by B-cell terminal differentiation into plasma cells (see section 1.3.3.1) (Schroder and Kaufman, 2005) (Reimold et al., 2001). We show that XBP-1s activated by non-physiological triggers, such as dithiothreitol (DTT), can induce KSHV reactivation (Wilson et al., 2007). This chapter also defines the mechanism by which XBP-1s induces reactivation of KSHV, and explores the possibility of a similar mechanism in related Rhadinoviruses. Finally, using the rKSHV.219 infected HEK 293T clonal cell lines and gene expression profiling we attempt to reveal other cellular regulators of KSHV lytic replication.

3.2 Results

3.2.1 rKSHV.219 infection of HEK 293T cells

The rKSHV.219 infected Vero cell line was obtained from the laboratory of Jeff Vieira. However, this kidney epithelial cell line derived from African green monkey (AGM) was not optimal for my work. This is predominantly due to the lack of infection by lentiviruses, a mechanism for gene delivery well established in the laboratory. Lentivirus infection is blocked by members of the tripartite motif (TRIMs) family of proteins, endogenous restriction factors expressed by AGM cells (Stremlau et al., 2004) (Keckesova et al., 2004). We therefore used the Vero cell line only for the initial production of recombinant rKSHV.219 virus.

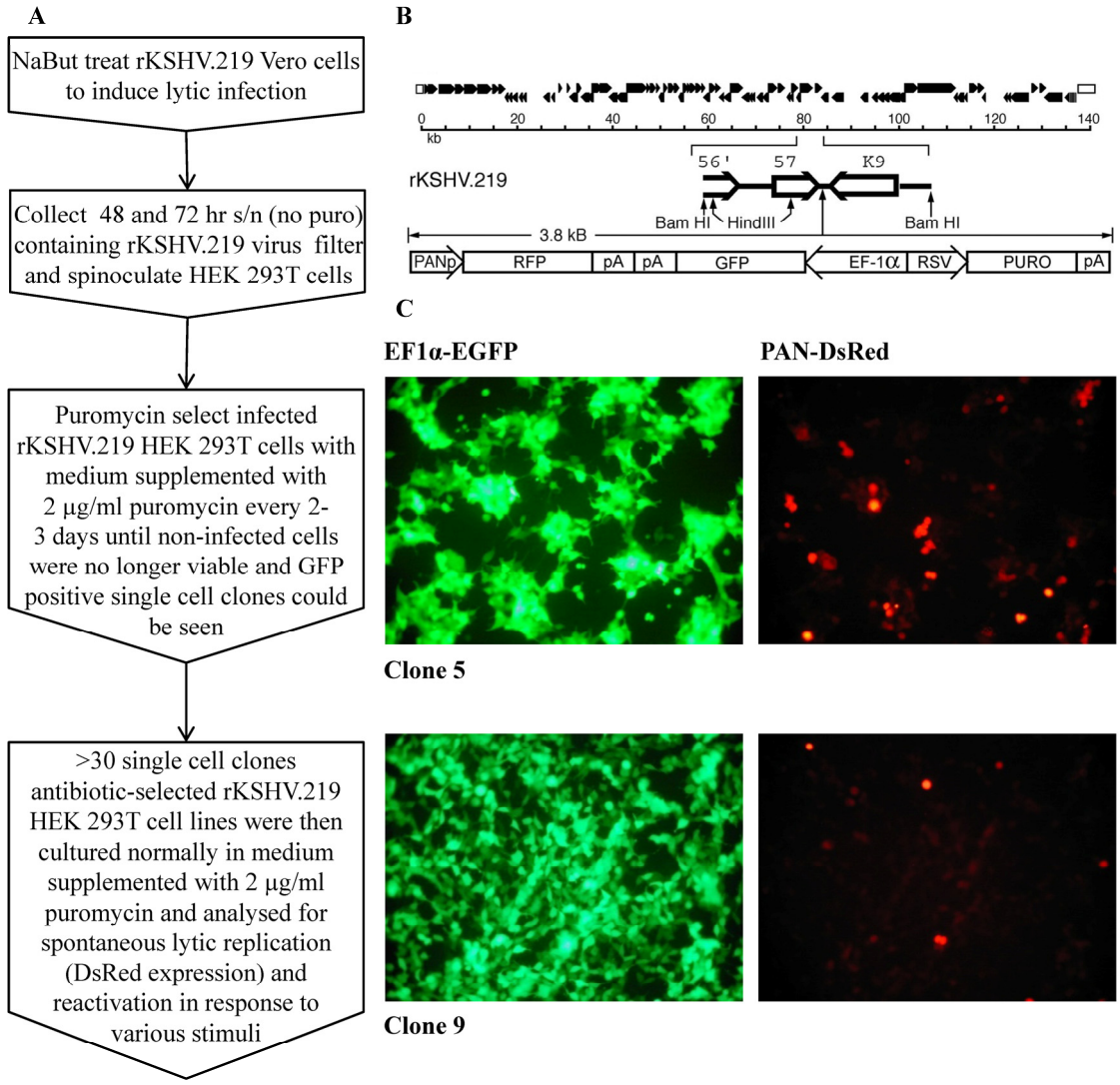

Figure 3-1 Generating rKSHV.219 HEK 293T cell lines
(A) Flow diagram summarising the method used to generate rKSHV.219 infected HEK 293T clonal cell lines. (B) Schematic diagram of the KSHV genome showing expansion of the 4.8 kb *BamHI* fragment used to make the recombinant rKSHV.219 virus, and the structure and insertion site of the RFP/GFP/PURO construct. The cassette is inserted between ORFK9 and ORF57 in a predicted non-coding region. The *BamHI* and *HindIII* sites of the 4.8 kb fragment

are indicated. Adapted from (Vieira and O'Hearn, 2004). (C) Photomicrographs of two morphologically distinct rKSHV.219 infected HEK 293T clonal cell lines with different levels of spontaneous reactivation. Typical fields of EF1α-GFP and PAN-DsRed fluorescence of each cell line are shown magnified at x20.

The method of generating stable rKSHV.219 infected HEK 293T cell lines is briefly summarised below and in Figure 3-1 A. Sodium butyrate was used to induce lytic reactivation in the original rKSHV.219 containing Vero cell line as described previously (Miller et al., 1997; Miller et al., 1996) (Vieira and O'Hearn, 2004). Infectious rKSHV.219 virus was collected in the cell-free culture medium of these cells and used to infect HEK 293T cells by spinoculation. HEK 293T cells are derived from human embryonic kidney (HEK) and were chosen for this system because they are permissive to infection, can be easily transfected and contain the SV40 large T antigen; to provide efficient and prolonged expression from plasmids containing an SV40 origin of replication. 48 hours after infection puromycin was used to select rKSHV.219 infected, GFP-positive HEK 293T single cell clones, which were expanded for further analysis. The clones obtained had mixed phenotypes, varying in morphology and in the amount of spontaneous lytic reactivation, represented by level of DsRed expression driven from the PAN promoter (Figure 3-1 B). Two examples of the rKSHV.219 infected HEK 293T cell lines generated are shown in Figure 3-1 C. The rKSHV.219 infected HEK 293T cell line, Clone 5, is an example of a clonal cell line that appears to clump and has a significant amount of spontaneous lytic replication. The cells of another clone, Clone 9, resemble regular HEK 293T cell morphology and represent a predominantly latent KSHV infection, with a spontaneous lytic population of approximately 1 %.

3.2.2 Characterisation of rKSHV.219 HEK 293T cell line – Clone 9

3.2.2.1 Response to various stimuli reported to reactivate latent KSHV

In order to characterise the rKSHV.219 infected HEK 293T cell lines generated and their potential use as a system to monitor the switch from latent to lytic KSHV replication, we studied their response to various factors known to induce reactivation. Starting with Clone 9, which displays a predominant latent infection with around a 1-3 % level of spontaneous lytic replication in untreated samples, similar to what is observed in the disease states of KS and PEL (Figure 3-2 A).

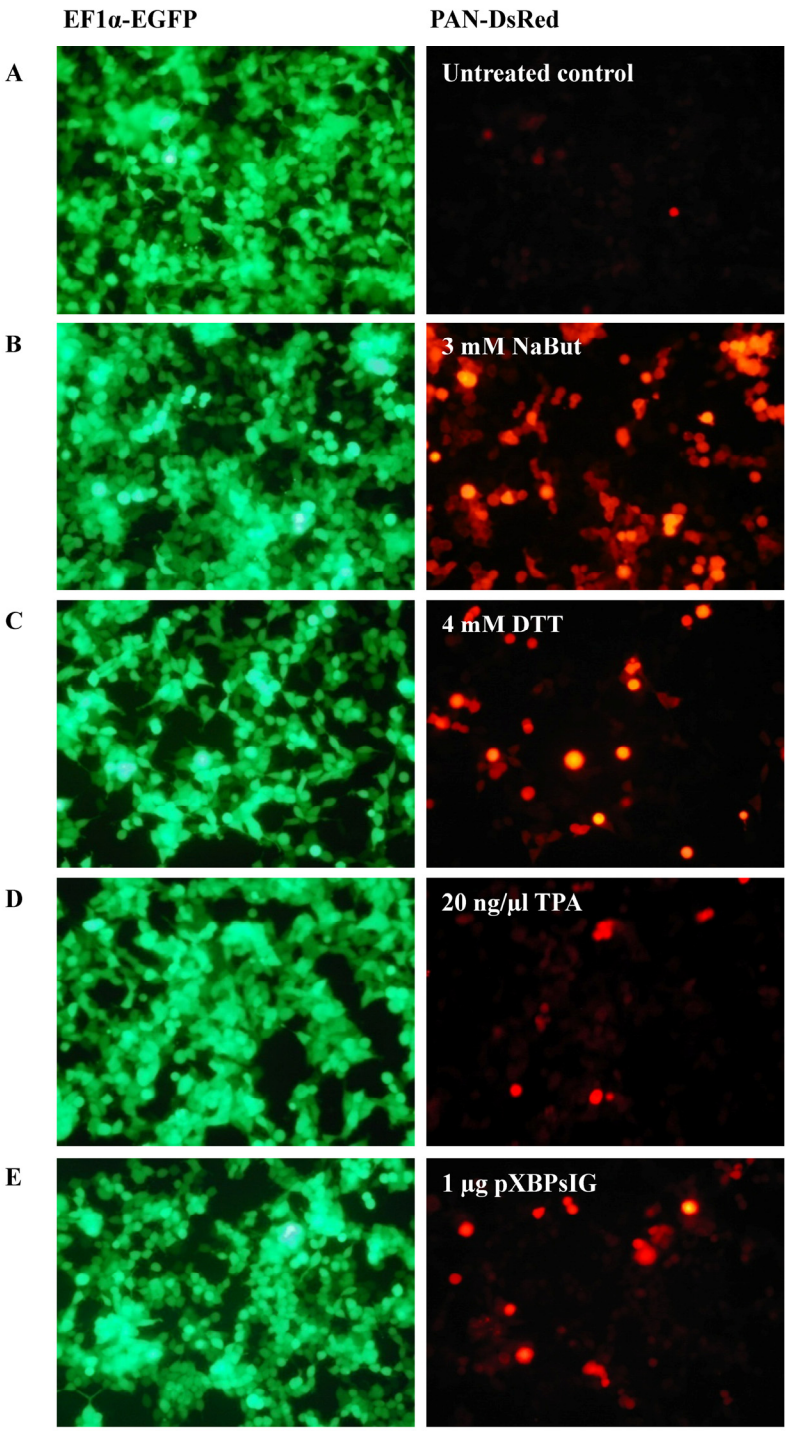

Figure 3-2 Treatment of Clone 9 cells with known inducers of KSHV reactivation
Photomicrographs of HEK 293T cells harbouring recombinant KSHV, rKSHV.219 (rKSHV.219 HEK 293Ts – Clone 9) 48 hours post-treatment. Typical fields of EF1α-GFP and PAN-DsRed fluorescence of each treatment magnified at x20 are shown. (A) Spontaneous levels of reactivation shown in untreated Clone 9 cells. Reactivation as demonstrated by DsRed expression in response to 3 mM sodium butyrate (NaBut) (B), 4 mM dithiothreitol (DTT) (C), 20 ng/µl TPA (D) or transfected with 1 µg pXBPsIG (E).

Treatment of Clone 9 cells with either 3 mM sodium butyrate (NaBut) or 4 mM DTT for 16 hours overnight led to significant reactivation of rKSHV.219, as measured by DsRed expression from the PAN lytic promoter 48 hours after

treatment (Figure 3-2 B and C). 48 hours after treatment with 20 ng/µl TPA low levels of DsRed expression were seen compared to untreated controls (Figure 3-2 D). Finally, overexpression of XBP-1 in its spliced active form induced KSHV reactivation as we have previously reported (Figure 3-2 E) (Wilson et al., 2007). Altogether this data shows that the rKSHV.219 infected HEK 293T cell line, Clone 9, faithfully represents a latent KSHV infection and responds to known triggers of lytic reactivation.

3.2.2.2 Dose dependent rKSHV.219 reactivation in response to DTT

To further analyse the rKSHV.219 infected HEK 293T cell line, Clone 9, the response to DTT, a known inducer of the UPR, over a range of concentrations was investigated. DTT is a reducing reagent that alters the redox environment of the endoplasmic reticulum (ER), and prevents the formation of di-sulphide linkages leading to the accumulation of misfolded proteins in the ER. Clone 9 cells were cultured in the presence of increasing concentrations of DTT for 16 hours first and then in fresh media for a further 48 hours before photomicrographs were taken, and the cells were prepared for FACs analysis. In response to DTT-induced stress Clone 9 cells displayed a dose dependent level of DsRed expression (Figure 3-3 A). The level of spontaneous lytic reactivation of Clone 9 cells was also measured in untreated control samples by FACsArray and was found to be below 3 % DsRed-positive (Figure 3-3 B).

Approximately 5 % of Clone 9 cells were driven into the KSHV lytic cycle after treatment with 4 mM DTT (Figure 3-3 B). To reach a final concentration of 4 mM DTT, 24 µl of DMSO containing DTT is added to 2 ml of media, this corresponds to 1.2 % of the total culture volume. This level of DMSO does not cause reactivation over and above the level of spontaneous reactivation 1-3 % measured by FACsArray (Figure 3-3 C). However the addition of larger volumes of DMSO, such as 45 µl (or 2.25 % of the total culture volume), causes levels of DsRed expression greater than background, and are therefore avoided (Figure 3-3 C). Higher concentrations of above 4 mM DTT appear to be toxic as fewer GFP-positive cells can be seen in these samples (Figure 3-3 A). Therefore in order to induce KSHV reactivation in Clone 9 cells specifically via DTT-induced UPR, without toxicity, the optimal concentration of DTT to be used is 3-4 mM.

When comparisons are made between the percentages of DsRed-expressing cells determined by FACsArray with the levels seen by microscopy of the same cells, we can see that the FACsArray underestimates DsRed levels compared to the fluorescent microscope (Figure 3-3 A and B).

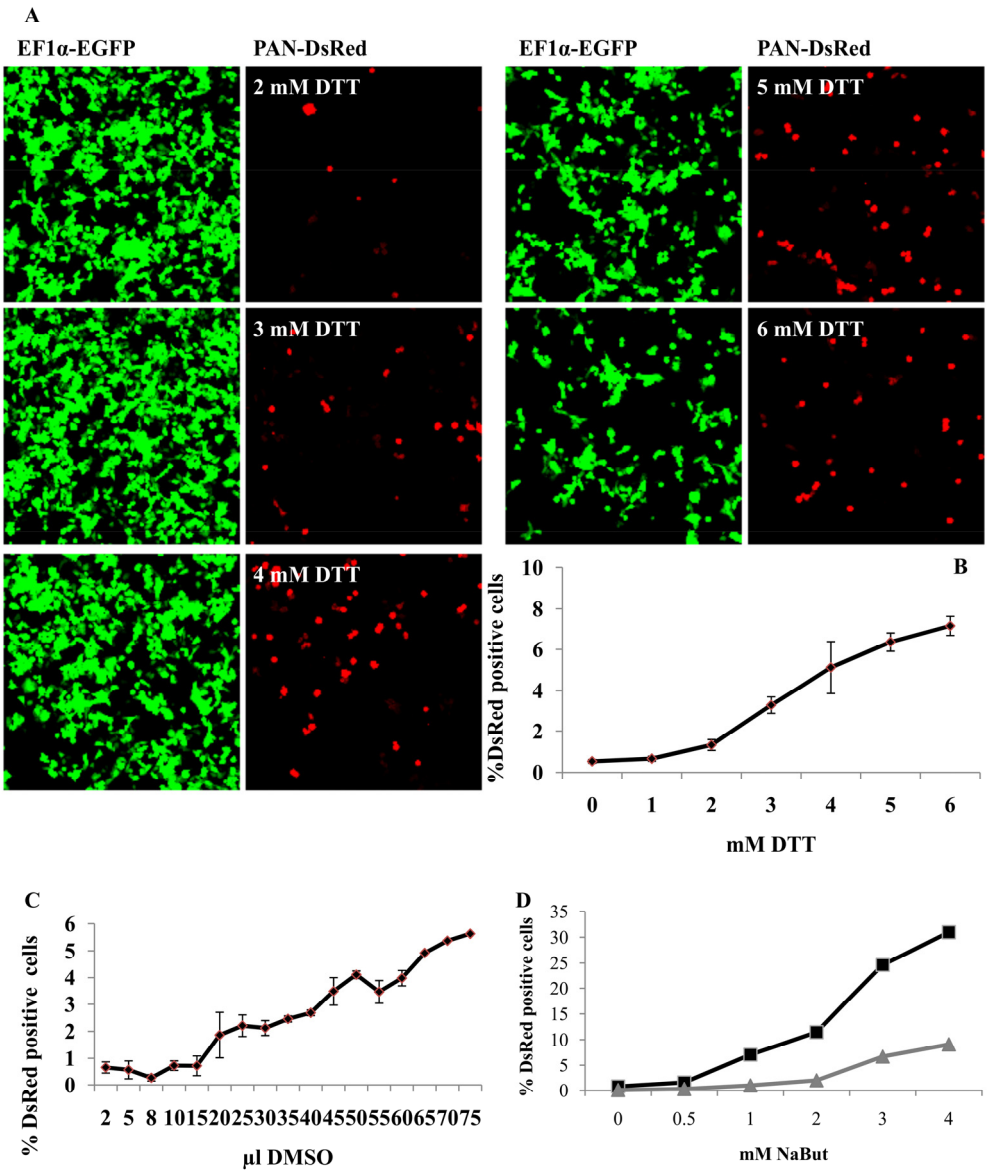

Figure 3-3 DTT titration on Clone 9 cells
(A) Photomicrographs of rKSHV.219 infected HEK 293T clonal cell line – Clone 9, 48 hours post-treatment with a range of DTT concentrations. Typical fields of EF1α-GFP and PAN-DsRed fluorescence of each condition magnified at x20 are shown. (B) Activity of the PAN lytic promoter in the cultures seen in the photomicrographs (A) were measured by flow cytometry and displayed as the percentage of DsRed-positive cells. Clone 9 cells displayed a dose dependent level of Ds-Red expression in response to DTT. (C) The percentage of DsRed-positive Clone 9 cells in response to a range of volumes of DMSO were measured by FACs. Volumes of DMSO below 40 µl do not induce DsRed expression above 2.5 %. Error bars represent the standard error of the mean of triplicate experiments. The percentage of DsRed cells resulting from 4mM DTT treatment is significantly different to those resulting from the equivalent amount of DMSO treatment $p<0.0274$ (D) The potential difference between the percentage of DsRed positive cells detected by the confocal microscope (squares) and FACs analysis (triangles). Clone 9 cells treated with NaBut were counted by automatic software before FACs analysis.

3.2.2.3 DsRed expression is dependent on RTA in Clone 9 cells

We have shown that the rKSHV.219 infected HEK 293T cell line, Clone 9, expresses DsRed in response to various known triggers of KSHV reactivation (section 3.2.2.1 and 3.2.2.2). In rKSHV.219 recombinant virus, DsRed expression is driven by the PAN lytic promoter, which is transactivated by RTA; an immediate early viral protein both necessary and sufficient for lytic replication (Vieira and O'Hearn, 2004) (Lukac et al., 1998). To confirm that in Clone 9 cells DsRed expression is dependent of RTA we took advantage of shRNA technology. Previously in the laboratory, a short hairpin RNA (shRNA) was designed to target RTA and shown to be effective at reducing the number of cells expressing RTA protein. An irrelevant control shRNA targeting GFP was also designed and shown to have no effect on RTA protein levels. Lentivirus expressing shRNA-RTA or control shRNA-GFP was used to transduce Clone 9 cells at an MOI 5. 48 hours post-transduction Clone 9 cells were then cultured in the presence or absence of 4 mM DTT overnight. Media was then replaced the next day and the DsRed expression was observed by confocal microscopy 24 hours later. Treatment of non-transduced Clone 9 cells with 4 mM DTT led to an increase in DsRed expression compared to control untreated cells (Figure 3-4 A). As expected GFP expression was noticeably lower in Clone 9 cells transduced with shRNA-GFP (Figure 3-4 B). DTT treatment of shRNA-GFP-expressing Clone 9 cells led to increased DsRed expression similar to non-transduced DTT treated samples (Figure 3-4 B). However, in the presence of shRNA-RTA DsRed expression was markedly reduced in response to DTT treatment compared to the non-transduced and shGFP transduced, DTT treated samples (Figure 3-4 C and A).

Additionally, non-transduced Clone 9 cells or Clone 9 cells expressing shRNA-RTA were cultured in the presence or absence of 3 mM NaBut. As expected, NaBut treatment of Clone 9 cells led to an increase in DsRed expression which was blocked by the presence of shRNA-RTA (Figure 3-4 D). Therefore, the DsRed expression detected in Clone 9 cells was directly due to KSHV reactivation, the presence of RTA and transactivation of the PAN lytic promoter. Importantly, we also demonstrate that shRNA mediated knock down of gene

expression is an effective way of dissecting the role of different proteins in Clone 9 cells.

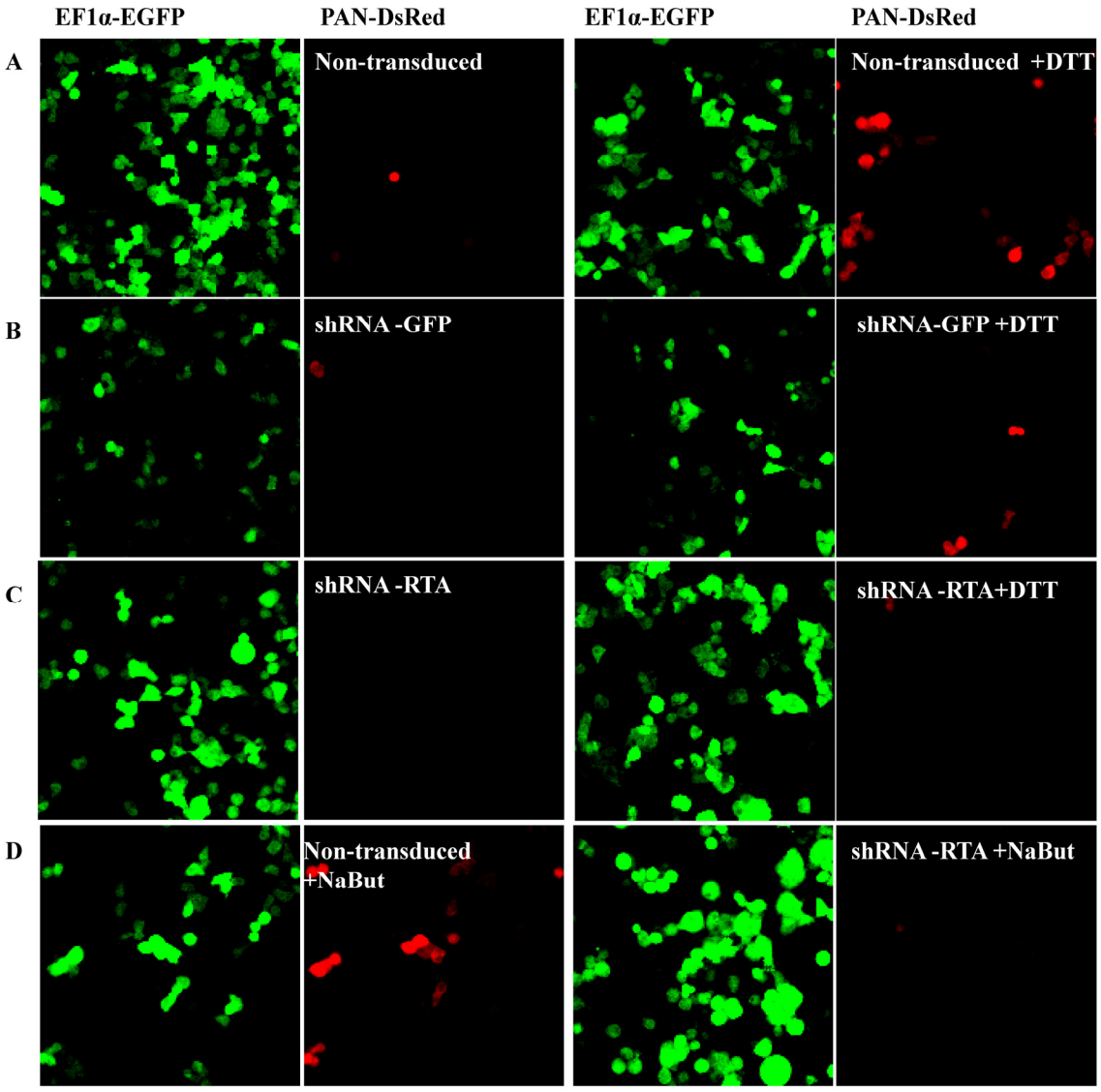

Figure 3-4 RTA dependence of DsRed expression in Clone 9 cells
rKSHV.219 HEK 293Ts – Clone 9 cells were transduced with lentivirus expressing shRNA-GFP or shRNA-RTA at MOI 5 and 48 hours later stimulated to induce the KSHV lytic cycle with 4 mM DTT or 3 mM sodium butyrate (NaBut) treatment. After 48 hours cells were analysed using confocal microscopy. Typical fields singly excited with 488-nm light to detect cells expressing GFP from the EF1α promoter (green) or 568 nm light to detect cells expressing DsRed from the KSHV lytic cycle PAN promoter (red) are shown magnified at x60. (A) Non-transduced Clone 9 cells treated with DTT have increased DsRed expression. (B) DsRed expression in response to DTT is also seen in the presence of shRNA-GFP. (C) DTT-induced DsRed expression is blocked by the presence of shRNA-RTA. (D) NaBut treated non-transduced Clone 9 cells have increased levels of DsRed expression relative to untreated controls which is blocked by the presence of shRNA-RTA.

3.2.3 Utilising the rKSHV.219 HEK 293T cell model for reactivation

Having characterised and established the rKSHV.219 infected HEK 293T cell line, Clone 9, we wished to use this model to further investigate the role of XBP-1s in the reactivation of KSHV (Wilson et al., 2007).

3.2.3.1 Effective knock down of XBP-1s protein using shRNA

It was previously observed that treatment of PEL cells with the drug DTT led to XBP-1 activation (XBP-1s). Also full KSHV lytic replication was induced by the overexpression of XBP-1s in latently infected cells (Wilson et al., 2007). To determine the direct involvement of endogenous XBP-1s in KSHV reactivation we therefore sort to remove XBP-1 from the rKSHV.219 system and monitor reactivation. A shRNA to target XBP-1 was previously designed (Figure 3-5 A) (Wilson et al., 2007). We showed that this hairpin was effective at reducing exogenous XBP-1s protein levels in HEK 293Ts cells stably expressing shRNA-XBP-1 (Figure 3-5 B). XBP-1 protein levels were unaffected by the presence of the control, shRNA-GFP (Figure 3-5 B). This validated a shRNA to XBP-1s, which we can use to study the function of XBP-1s in the induction of KSHV lytic replication.

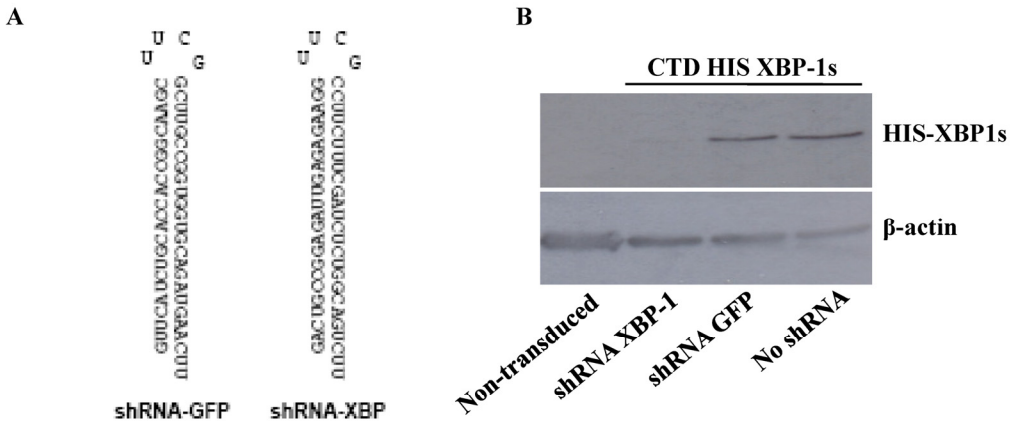

Figure 3-5 Validation of shRNA-XBP-1
(A) Sequences and probable structures of shRNAs targeting GFP or XBP-1 respectively. (B) shRNA ability to knock down target protein levels was demonstrated by co-transfection of HEK 293T cells with 1 µg of C-terminal domain (CTD) HIS tagged XBP-1s-expressing plasmid (CTD HIS XBP-1s) and 3 µg of relevant/irrelevant control hairpins in a pGEMT backbone. Western blot using anti-HIS for HIS-tagged XBP-1 detection. β-actin acts as a loading control.

3.2.3.2 Endogenous XBP-1s results in rKSHV.219 reactivation from latency in Clone 9 cells

Using shRNA to XBP-1 and rKSHV.219 HEK 293T, Clone 9 cells, we investigated whether DTT-mediated endogenous IRE1 activity results in induction of KSHV lytic replication through XBP-1s. Since Clone 9 cells enter the lytic cycle after exposure to 4 mM DTT, we first looked to see if this DTT treatment also led to the induction of XBP-1s processing, consistent with the data derived in PEL cells (see section 3.2.2.2.) (Wilson et al., 2007). XBP-1 transcript splice status was monitored using a modified version of an RT PCR assay developed by Heather Harding, (Calfon et al., 2002) (Figure 3-6 A). Minimal XBP-1 spliced transcript is present in Clone 9 cells maintained in growth medium without DTT treatment (Figure 3-6 B). A 4 mM DTT overnight treatment of Clone 9 cells resulted in the detection of a strong band representing spliced XBP-1 that could not be seen in untreated controls (Figure 3-6 B).

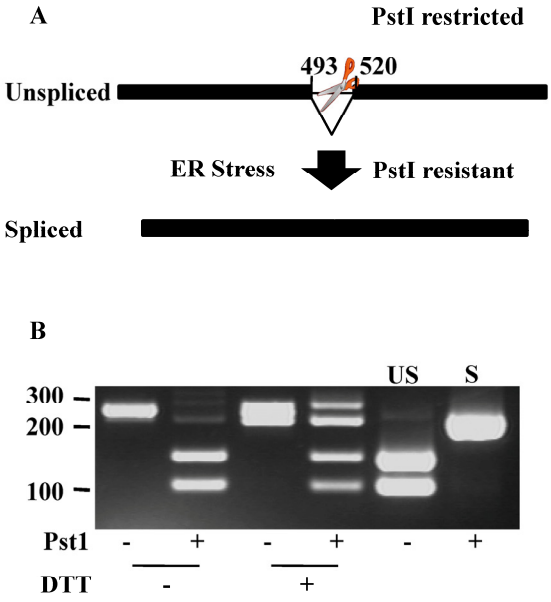

Figure 3-6 DTT induces XBP-1 splicing in Clone 9 cells
(A) Schematic representation of XBP-1 mRNA processing. Nucleotides are numbered relative to translation initiation. The PstI restriction site located in the intronic region is lost during a splicing event. Unspliced XBP-1 is therefore digested by PstI whereas spliced XBP-1s is resistant to digestion by PstI (B) RT PCR amplification across the XBP-1 intron produces a 249 base pair amplicon from XBP-1u mRNA and a 223 base amplicon from XBP-s mRNA. Pst I digests only the XBP-1u amplicon resulting in two bands, whereas XBP-1s results in a single band (Wilson et al., 2007). RT PCR amplification from the total mRNA of Clone 9 cells untreated or treated with DTT. A band corresponding to spliced XBP-1 is seen in DTT treated Clone 9 cells, a slower-migrating non-Pst I digestible PCR hybrid between the XBP-1s and XBP-1u products is visible in this lane as previously described (Wilson et al., 2007). RT PCR for β-actin on the same RNA was used to normalise cDNA input (data not shown).

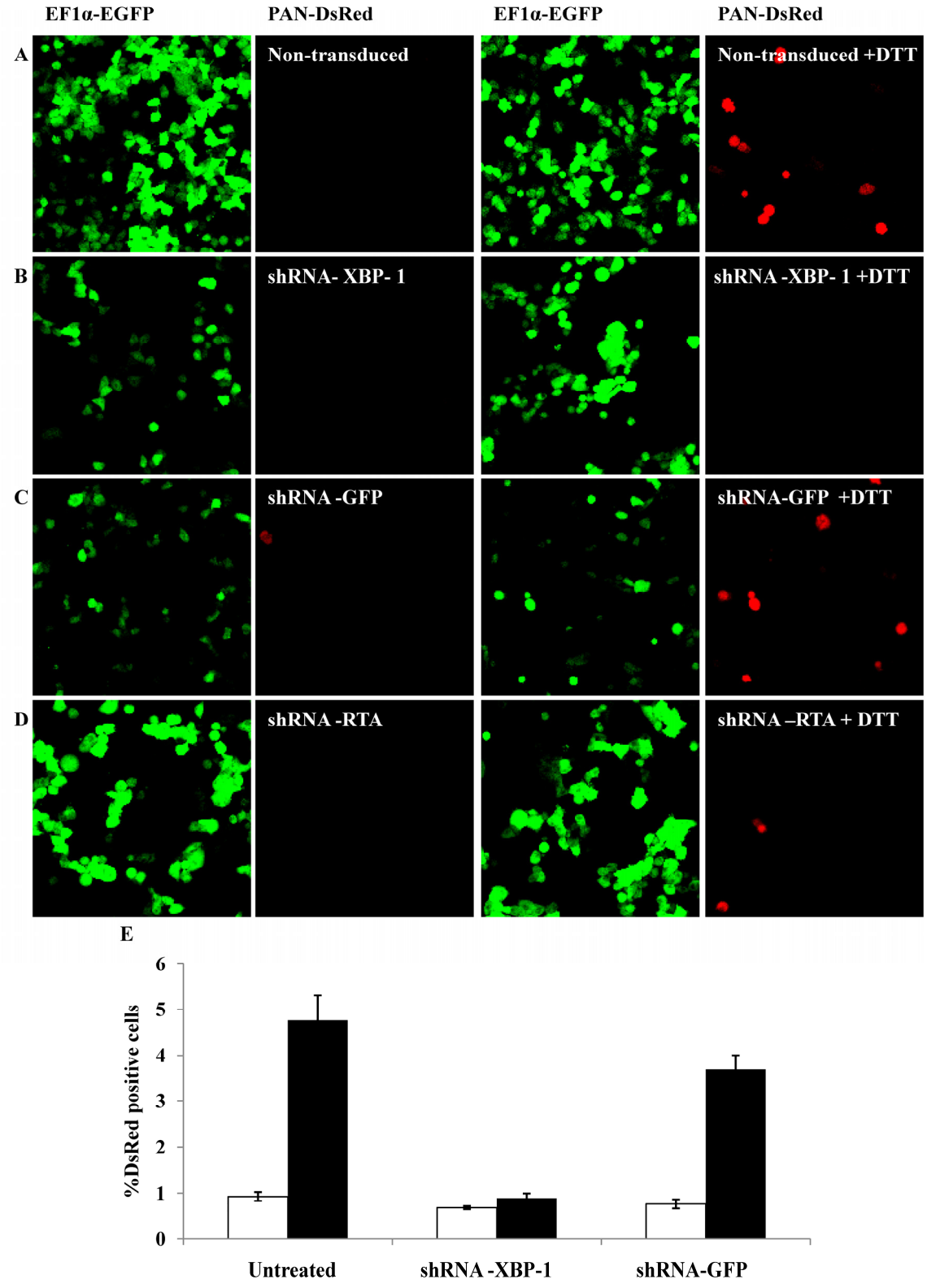

Figure 3-7 Endogenous XBP-1s induces rKSHV.219 reactivation in Clone 9 cells
(A) HEK 293T cells harbouring recombinant KSHV, rKSHV.219 (Clone 9 cells), were stimulated to induce the KSHV lytic cycle by 4 mM DTT treatment overnight. At 48 hours post-treatment, cells were analyzed using confocal microscopy as described in Figure 3-3. Clone 9 cells were also transduced at an MOI 5 with lentiviral vectors expressing shRNA XBP-1 (B), shRNA GFP (C), or shRNA-RTA (D) and treated with DTT as described above. (E) Activity of the PAN lytic promoter in the cultures seen in the confocal images (A-C) were measured by flow cytometry and displayed as the percentage of DsRed-positive cells. Columns represent mean of three independent experiments and error bars represent the standard error of the mean.

As seen before in section 3.2.2.2, increased DsRed expression indicating KSHV lytic cycle initiation was detected 48 hours after DTT treatment of non-transduced Clone 9 cells (Figure 3-7 A). The percentage of DsRed-positive non-transduced Clone 9 cells after DTT treatment as determined by FACsArray was 4.7 % (Figure 3-7 E). However, the photomicrograph of the same cells shows that the proportion of DsRed-positive cells was again underestimated by the FACsArray (Figure 3-7 A).

Importantly, the reactivation seen in Clone 9 cells in response to DTT is blocked by the presence of a shRNA specific for XBP-1 but not by an irrelevant shRNA targeted to GFP. This is clearly visible when photomicrographs of DTT treated shRNA-XBP-1-expressing Clone 9 cells (Figure 3-7 B) are compared to DTT treated, non-transduced or shRNA-GFP-expressing cells (Figure 3-7 A or C). The percentage of DsRed-positive cells in DTT treated shRNA-GFP transduced samples was quantified by FACsArray as 3.7 % which was reduced to 0.89 %, background levels, in shRNA-XBP-1-expressing Clone 9 cells after DTT treatment (Figure 3-7 E). As a further control for transduction, as well as DTT induction of the lytic cycle via RTA expression, shRNA-RTA was included in the experiment. As seen previously the presence of shRNA to RTA led to the reduction of DsRed expression in Clone 9 cells after DTT treatment (Figure 3-7 D). This data provides evidence that endogenous activation of XBP-1 by DTT-induced UPR can specifically cause KSHV reactivation from latency.

3.2.4 The ORF50 promoter and XBP-1 response elements

3.2.4.1 The KSHV ORF50 promoter contains a novel XBP-1 responsive element

We demonstrated that the highly active plasma cell transcription factor XBP-1 is a lytic switch trigger for KSHV using rKSHV.219 HEK 293T, Clone 9 cells. Previously, we showed that only expression of the spliced active isoform of XBP-1 (XBP-1s) in PEL cell lines robustly increased the expression of RTA, and this was sufficient to initiate the full lytic cycle (Wilson et al., 2007). The direct action of XBP-1s on the RTA promoter was also mapped to the 200 base pairs preceding the RTA start codon (Wilson et al., 2007). Examination of the targets for XBP-1 binding previously identified by chromatin immunoprecipitation (ChIP)

(Acosta-Alvear et al., 2007), revealed a potential XBP-1 response elements (XRE) in the ORF50 promoter with an 'ACGT' core containing motif (Figure 3-8 A). To assess the relevance of this we determined the effect of XBP-1s expression on a luciferase reporter gene cloned downstream of the wild type ORF50 promoter and an ORF50 promoter sequence containing a mutant putative XRE in transient transfection assays (Figure 3-8 A). Both the wild type and mutant promoters responded to overexpressed RTA (Figure 3-8 B). Overexpression of XBP-1s was able to activate transcription from the wild type promoter with a 10 fold increase in activity with respect to empty vector control (pIG). However, XBP-1s overexpression failed to transactivate the mutant ORF50 promoter (Figure 3-8). This data identifies a functional XBP-1 response element 76 base pairs upstream of the starting methionine in the ORF50 promoter. This element was previously identified as a weak HIF-1α response element 4 (HRE4) in the ORF50 promoter (Cai et al., 2006a) and we propose this element should be renamed XRE (Dalton-Griffin et al., 2009).

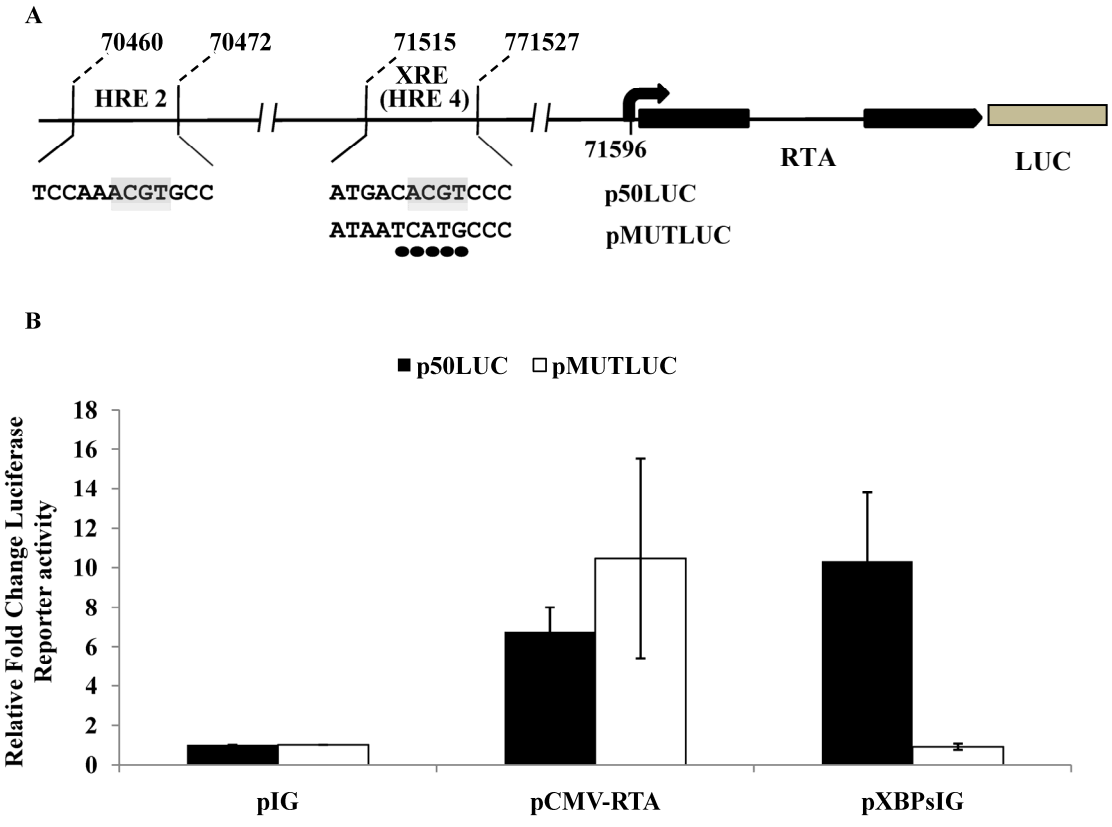

Figure 3-8 A novel XBP-1s response element (XRE) in the ORF50 promoter
(A) A schematic representation of the ORF50 promoter and coding region included in the luciferase reporter, showing the location of the predominant hypoxia response element (HRE2) as well as the newly identified XBP-1 response element (XRE), previously identified as the putative HRE4. Adapted from (Cai et al., 2006a). Numbers in brackets indicate the response element start sites relative to KSHV genome sequence (NC_003409). The wild type sequence of both HRE2 and XRE are shown with the core 'ACGT' sequence element highlighted in grey.

The mutated sequence of the XRE is indicated underneath with mutated nucleotides indicated by (•). (B) HEK 293T cells were transfected with a *Renilla*-expressing control plasmid (phRL-null) plus the *Firefly* luciferase reporter plasmid, with either the wild type (p50LUC - black bars) or the mutant (pMUT50LUC - white bars) ORF50 promoter sequence together with pIG (control), RTA-expressing (pCMV-RTA) or XBP-1s-expressing (pXBPsIG). *Firefly* luciferase activity was determined 48 hours post-transfection and normalised to *Renilla* luciferase (phRL-null) activity and plotted as a fold change relative to empty vector control pIG. Columns represent mean of three independent experiments and error bars represent the standard error of the mean.

3.2.4.2 Establishing the ORF50 promoter regions for other Rhadinoviruses

Having established a mechanism of KSHV reactivation that is linked to B-cell terminal differentiation, we were keen to assess whether the mechanism was potentially conserved among other Rhadinoviruses and their hosts. This seemed plausible due to the fact that the UPR is highly conserved from yeast to mammals and RTA is highly homologous across Rhadinoviruses (Ron and Walter, 2007) (Damania et al., 2004). To address this we attempted to identify potential XREs in the ORF50 promoter regions of several Rhadinoviruses. The RTA transcripts of Rhadinoviruses have also been shown to have similar structures, consisting of a small first exon followed by an intron and a second larger exon (Lukac et al., 1999) (Sun et al., 1998) (DeWire et al., 2002) (Wu et al., 2000b). Due to the small size of the first exon it is often missing from annotations and deposited sequences. We therefore sort to determine the correct start sites for the RTA coding regions of different Rhadinoviruses before searching for potential XREs.

This was achieved by using the KSHV ORF50 intronic sequence as a template to BLAST against published Rhadinoviruses complete genome sequences within the NCBI sequence database. When significant matches were found the first exon could easily be identified by searching for the nearest and smallest open reading frame upstream of this putative intron. All prospective transcripts were checked for functional splicing elements by identifying splice donor and acceptor sites. Schematic diagrams of the full ORF50 transcripts for KSHV, Macaca mulatta Rhadinovirus (RRV), Ateline herpesvirus (HVA), Herpesvirus Saimiri (HVS) and Murine herpesvirus 4 (MHV 68) are shown (Figure 3-9). Numbering refers to the genomic sequences with accession numbers indicated.

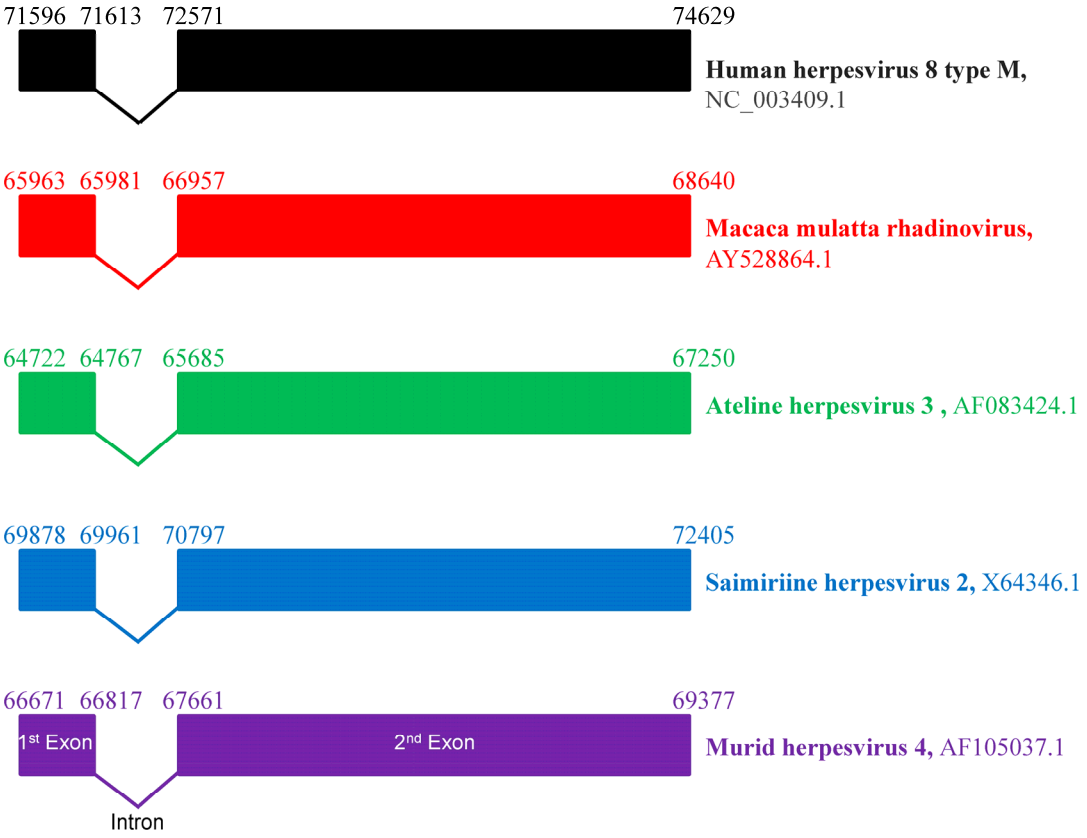

Figure 3-9 ORF50 promoter regions for other Rhadinoviruses
Schematics denote first exon, intron and second exon of RTA homologues. Numbering relates to genome position as given by the NCBI sequence database. All transcripts are spliced efficiently.

3.2.4.3 Identifying the potential XBP-1 response elements in other Rhadinovirus ORF50 promoters

A genome wide search for genes regulated by the transcription factor XBP-1 revealed that XBP-1 is involved in various functionally distinct processes and acts through different motifs or response elements (Acosta-Alvear et al., 2007). Using this information, we identified a functional XRE in the KSHV ORF50 promoter (see section 3.2.4.1) (Dalton-Griffin et al., 2009). Next, we examined the first 500 nucleotides upstream of the transcriptional start site of ORF50 in several Rhadinoviruses for similar, potential XBP-1 binding sites as predicted by Acosta-Alvear (Figure 3-10 A). XREs are indicated by the presence of the core sequences, 'ACGT', 'CGTG' and 'ACGC/A' (Figure 3-10 B) (Acosta-Alvear et al., 2007). A sequence logo was created to apply weight to each nucleotide found within a potential XBP-1 binding site, providing a consensus sequence for XREs in the Rhadinovirus ORF50 promoter (Figure 3-10 C). This analysis identifies potential XREs present within the promoter regions of ORF50 from several Rhadinoviruses; further work is required to determine their functionality.

A

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ACGCTAGGGTCTCCCCACCCAACCCCCATAGGACCCAGCTACAGCTTATCCTCCACTAAATACCAGGCAGCTACCGG
CAGCCATGACACCGTGAACTTCCTGATGTCTCCTAAAACTAACCCCGAAACAGAGCTAAATACCAATGACTGTCACC
TACTGAAAGCTCCATGAGTAAACTAAATACTTTATCCCACACTAACCAGTTAAATACAGAGCAAGTTGTTTACGCCC
TGGCAATGAAAGCTCCATAGTCAAGCTAAATGCTATCATCAACTAACTAGTTAAATACCATGCAGTTGTTTCCACCC
TCAAGGTCCATTTTTGAACACATTATCCCACAATGTGCTGCAGTCTGCAAGAAATAATAGCCTCCCACTTTTATGGA

CGACTCATTAAGCCCCGCCCAGAAACCAGTAGCTGGGTGGCAATGACACGTCCCCTTTAAAAAGTCAACCTTACTCC
CCTACCCCAAGCCACGCCCCCGTACTATTAGACCAGGGTGAGTAACCACGCTATCCTTTAAAAACCCATACGTGGAG
CCTAAACACACCCACCGCTGACTAAGCTAAATGCCCTAAAGCATTTAAAGGGAAGACTTTACATAGTGTACCACCAC
ATAGACACGCCTACCTCATTAATAAGCTAAATGCCCTGAAGCATTTAAAAGGAAGAATTTACATAGTGTACAATCAC
AATCTGGTAGCTCCTCCTACATGAGTCATGAAATGTCCCTTCAAAGGCTGAGGAGAGAGCACAACTCTTTTCAATCT

GCAAGGGGTAGTCTGTTGTGAGAATACTGTCCAGGCAGCCACAAAAATG Human herpesvirus 8 type M, complete genome NC_003409.1
TTTGTAAGGTAAACAGCTCGTGTATATTTCAGACGCCTGTCATAAAATG Macaca mulatta rhadinovirus, complete genome AY528864.1
TCTTCACAAACTTGGAATCTTACCTTTTAACTCCACAAATACAACAATG Ateline herpesvirus 3 complete genome AF083424.1
TCTGCACAAACTTGAGATCTTACAGCTGAGCTCTTAAGCAACAACAATG Saimiriine herpesvirus 2 complete genome X64346.1
ACACTCAAAAATACAACTCCTGCACTACGAGGGAATTTCTGCAGCGATG Murid herpesvirus 4 complete genome AF105037.1
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B

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GAC | ACGT | CCC Human herpesvirus 8 type M, NC_003409.1
ACA | CCGT | GAA
GCC | ACGC | CCC
ACC | ACGC | TAT Macaca mulatta rhadinovirus, AY528864.1
CAT | ACGT | GGA
AGC | TCGT | GTA
CAG | ACGC | CTG
TTT | ACGC | CCC Ateline herpesvirus 3 AF083424.1
GAC | ACGC | CTA Saimiriine herpesvirus 2 X64346.1
ACT | ACGA | GGG Murid herpesvirus 4 AF105037.1
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C

Figure 3-10 Potential XBP-1 response elements in the first 500 nucleotides upstream from the translation start site of selected Rhadinovirus ORF50 promoters
(A) Manual alignment of the first 500 bp of the promoter regions of the ORF50 genes of Human herpesvirus 8, Macaca mulatta Rhadinovirus, Ateline herpesvirus 3, Saimiriine herpesvirus 2 and Murid herpesvirus 4, by anchorage to the starting ATG in each case. Potential XBP-1 binding sites are highlighted. (B) Representation of the XBP-1 core binding sites and surrounding sequences identified within the promoter regions. (C) Sequence logo of consensus XBP-1 response element, size of letter determines how often that nucleotide is seen at that position within the elements. Created at http://weblogo.berkeley.edu/logo.cgi

3.2.5 Characterisation of rKSHV.219 HEK 293T cell line – Clone 5

Infection of HEK 293T cells with rKSHV.219 gave rise to a number of cell clones that were morphologically different and varied in the amount of spontaneous DsRed expression. One of these clones, Clone 5, appeared to form cell clumps as well as display higher levels of spontaneous DsRed expression compared to those seen in Clone 9 cells. We therefore sort to characterise Clone 5 cells in relation to Clone 9 cells to find a possible explanation for the augmented level of reactivation.

3.2.5.1 The response of Clone 5 cells to DTT treatment

Clone 9 cells were previously shown to respond to DTT treatment with a dose dependent level of reactivation (see section 3.2.2.2). In order to characterise Clone 5 cells we also assessed the DsRed expression in response to DTT. Clone 5 and Clone 9 cells were cultured for 16 hours, overnight, in the presence or absence of 3 mM DTT. DTT was removed the following day and the level of DsRed expression was recorded by light microscopy a further 24 hours later.

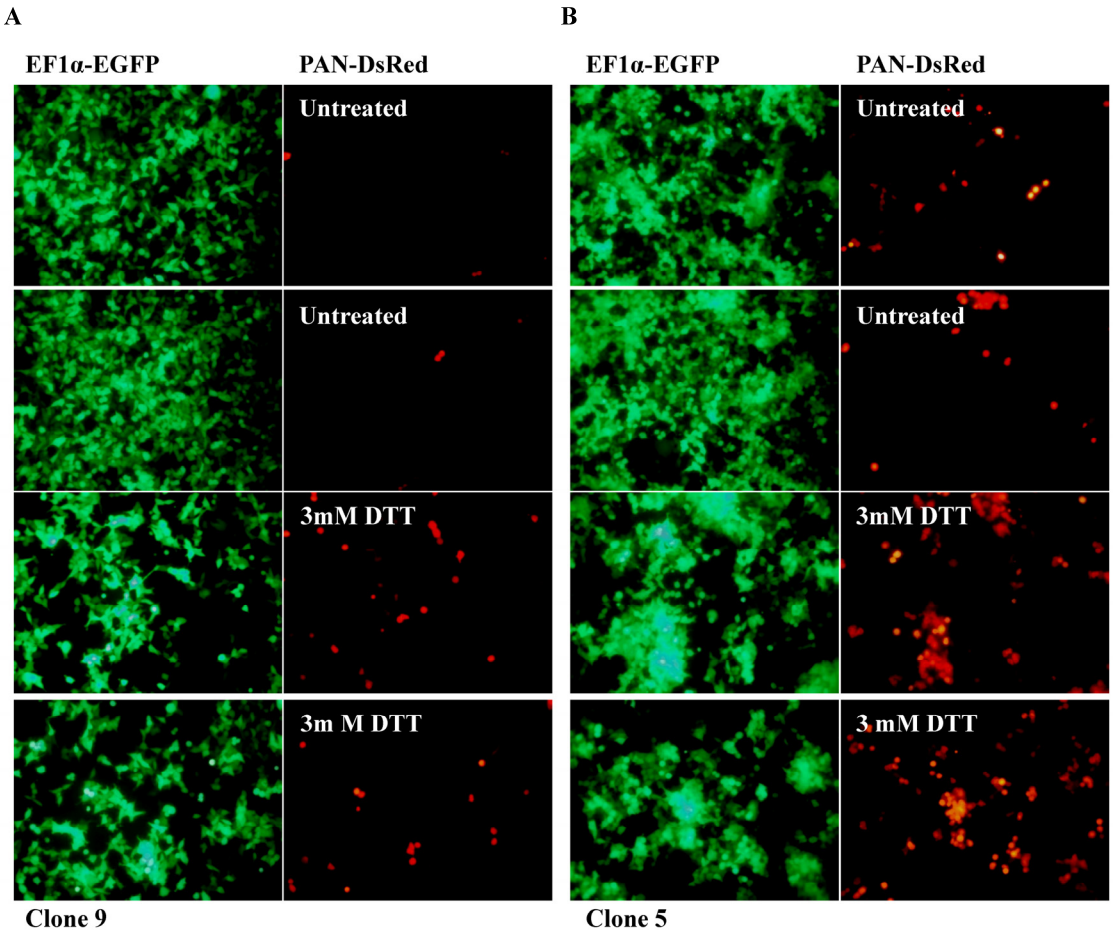


Figure 3-11 DTT treatment of Clone 5 cells

Photomicrographs of rKSHV.219 infected HEK 293T clonal cell lines – (A) Clone 9 or (B) Clone 5 taken 48 hours after 3 mM DTT treatment overnight. Two typical fields of EF1α-GFP

and PAN-DsRed fluorescence of each condition untreated or treated with 3 mM DTT for each cell line magnified at x20 are shown. Clone 5 cells display more spontaneous DsRed expression. DsRed expression increases in response to DTT in both Clone 5 and Clone 9 cells.

As demonstrated in section 3.2.2.2, DTT treatment of Clone 9 cells induces DsRed expression greater than the spontaneous levels seen in untreated controls (Figure 3-11 A). The higher level of spontaneous DsRed expression observed in Clone 5 cells can be seen when comparing untreated Clone 5 cells in Figure 3-11 B with those of untreated Clone 9 cells in Figure 3-11 A. DsRed expression in Clone 5 cells was also further increased in response to DTT treatment (Figure 3-11 B). Together, this shows that Clone 5 cells have an enhanced level of spontaneous DsRed expression compared to what is observed for Clone 9 cells, and that DTT treatment further induced DsRed expression in both cell lines.

The difference in morphology between Clone 9 and Clone 5 cells is seen most clearly when DTT treated samples from Figure 3-11 A and B are compared. Clone 9 cells retained the evenly spaced, spindle-like morphology of HEK 293T cells whereas Clone 5 cells had a tendency to form large clumps. This phenotype was less easily seen when cells were more confluent.

3.2.5.2 XBP-1 activity and the high level of rKSHV.219 reactivation in Clone 5 cells

DTT is a known inducer of the UPR and a major component of this response is the transcription factor XBP-1. We have shown that XBP-1 is activated by a splicing event that can be induced by DTT treatment, and results in KSHV reactivation (see section 3.2.3.2) (Wilson et al., 2007). Also exogenous expression of XBP-1s in the rKSHV.219 HEK 293T cell line, Clone 9, drives DsRed expression which corresponds directly to reactivation (see section 3.2.2.1). We therefore wanted to determine if the higher spontaneous DsRed expression observed in Clone 5 cells was perhaps due to an augmented XBP-1 activity compared to Clone 9. Semi-quantitative RT PCR for the presence of XBP-1 transcript shows that both Clone 9 and 5 cell lines have similar undetectable resting levels of XBP-1s. After overnight DTT treatment the level of XBP-1s transcript in Clone 9 and Clone 5 cells increased to similar levels (see section 3.2.3.2) (Figure 3-12 A). Therefore untreated and DTT treated

Clone 5 cells do not have significantly higher levels of active XBP-1s transcript than Clone 9 cells. The increased spontaneous DsRed expression seen in Clone 5 cells is therefore not due to increased levels of XBP-1s.

In parallel samples obtained 48 hours after DTT treatment, we monitored the relative levels of rKSHV.219 lytic induction using RT PCR for the spliced late lytic gene ORF29a/b (Renne et al., 1998). In untreated Clone 9 cells, where spontaneous reactivation is less than 1 %, ORF29a/b was undetectable (Figure 3-12 B). After DTT treatment a slight increase in lytic transcript can be seen confirming the ability of DTT to induce KSHV reactivation in Clone 9 cells (Figure 3-12 B). In agreement with the increased level of spontaneous DsRed expression in Clone 5 cells, ORF29a/b transcript was detected in resting cells (Figure 3-12 B). DTT treatment of Clone 5 cells significantly induced ORF29a/b transcript, and to levels greater than those in Clone 9 cells after DTT treatment (Figure 3-12 B). RT PCR for β-actin was used to normalise for cDNA input and provides semi-quantitative data for both XBP-1 and ORF29a/b RT PCR (Figure 3-12 C). Together, these data show that higher levels of DsRed expression observed in Clone 5 compared to Clone 9 cells correlate with higher levels of lytic reactivation, as measured by ORF29a/b transcript, and which is not linked to higher levels of XBP-1 splicing in these cells.

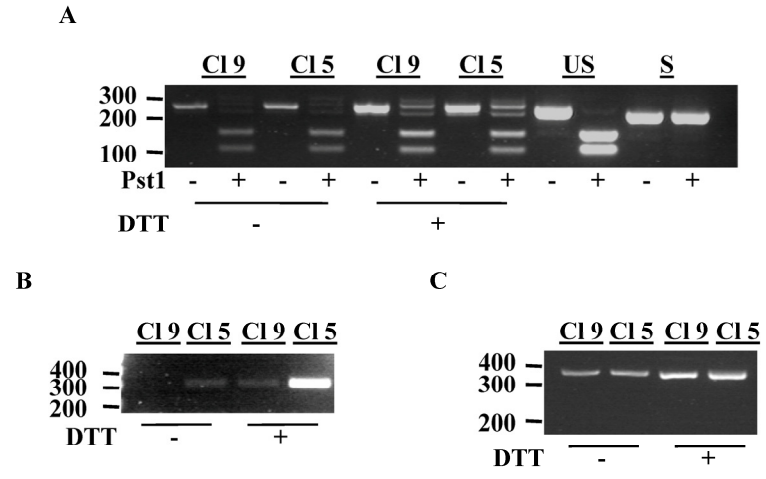


Figure 3-12 XBP-1 splice status of Clone 5 cells
(A) RT PCR amplification from the total mRNA of Clone 5 and Clone 9 cells; untreated or treated with DTT. PstI non-restricted bands of amplified XBP-1s product are visible in the DTT treated samples only, and at similar levels. (B) A 300bp product was amplified from RNA samples taken 48 hours after DTT treatment with gene specific primers for the spliced lytic transcript, ORF29 a/b. PCR product is seen in untreated Clone 5 and in samples with XBP-1s after DTT treatment corresponding to the induction of KSHV lytic cycle and the DsRed seen in Figure 3-11. (C) RT PCR for β actin on the same RNA was used to normalise cDNA input.

3.2.5.3 DsRed expression is dependent on RTA in Clone 5 cells

To further conclude that the higher levels of spontaneous and DTT-induced DsRed expression in Clone 5 cells were due to increased RTA expression and lytic replication; cells were transduced with lentivirus expressing shRNA-GFP or shRNA-RTA at an MOI 5. 48 hours after transduction, cells were cultured in the presence or absence of 4 mM DTT for 16 hours to induce the lytic cycle. Cells were then cultured in fresh media for 48 hours before representative photomicrographs of the cells were taken and cells were sampled for both western blot analysis and RNA extraction followed by cDNA synthesis.

Expression of shRNA-RTA in Clone 5 cells alone led to a reduction in the spontaneous DsRed expression seen when compared to Clone 5 cells transduced with the control shRNA targeted to GFP (Figure 3-13 A and B). After lytic cycle induction with DTT, DsRed expression was increased in Clone 5 cells transduced with shRNA-GFP as expected (see section 3.2.5.1) (Figure 3-13 A). Although DsRed expression did increase after DTT treatment of Clone 5 cells expressing shRNA-RTA, this was clearly repressed in contrast to the shRNA-GFP-expressing DTT treated sample (Figure 3-13 B and A). Western blot analysis for RTA expression also reveals low levels in resting Clone 5 cells that are robustly increased in response to DTT treatment (Figure 3-13 C). Therefore, RTA is required for the DsRed expression following addition of DTT to Clone 5 cells.

To confirm that lytic cycle induction goes beyond RTA expression in Clone 5 cells, we looked at lytic gene ORF29a/b expression in these samples, and in samples treated in an identical way but with Sodium Butyrate as the inducing agent (Figure 3-13 D). The semi-quantitative RT PCR reveals that the known triggers of KSHV reactivation, DTT and NaBut, increased the presence of the spliced ORF29a/b transcript in the control shRNA-GFP-expressing Clone 5 cells. In the presence of shRNA-RTA the levels of RTA induced by DTT and NaBut exposure should be effectively reduced and therefore should impair lytic replication initiation. Levels of ORF29a/b transcript were marginally increased in DTT or NaBut treated Clone 5 cell samples transduced with shRNA-RTA but were lower than those seen in samples expressing irrelevant control shRNA (Figure 3-13 D). Input levels of cDNA were normalised using RT PCR for β-actin

(Figure 3-13 D). The DsRed expression in Clone 5 cells faithfully represents lytic reactivation of the rKSHV.219 in these cells, and the presence of RTA is required for both the spontaneous and induced levels of DsRed seen.

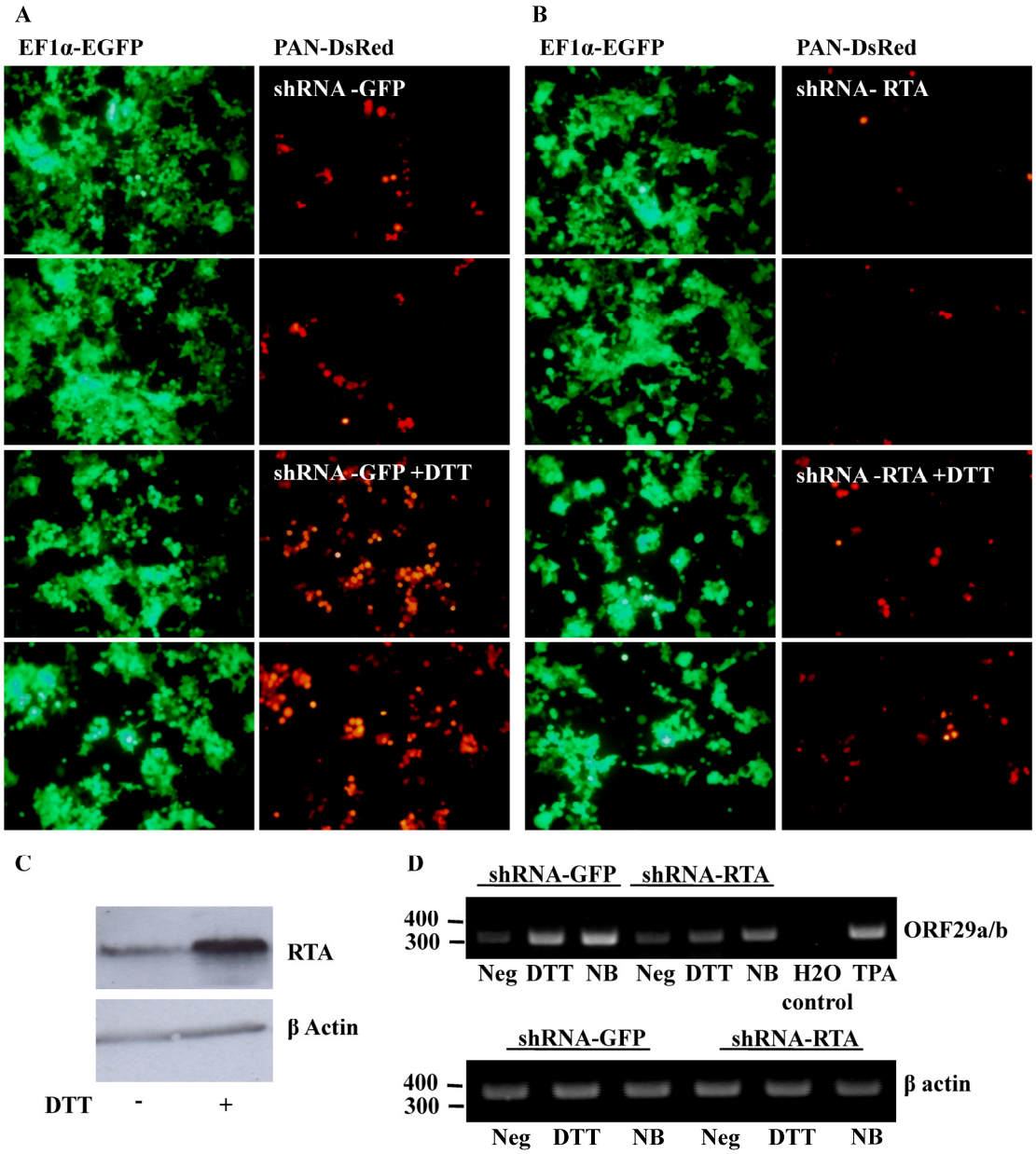


Figure 3-13 RTA dependence of DsRed expression in Clone 5 cells
rKSHV.219 HEK 293Ts, Clone 5 cells were transduced with lentivirus expressing (A) shRNA-GFP or (B) shRNA-RTA at an MOI 5 and 48 hr later stimulated to induce the KSHV lytic cycle with 4 mM DTT treatment. After 48 hours cells photomicrographs were taken. Two typical fields of EF1α-GFP and PAN-DsRed fluorescence of each shRNA-expressing cell line untreated or treated with 4 mM DTT magnified at x20 are shown. (A) shRNA-GFP-expressing Clone 5 cells treated with DTT have increased DsRed expression. (B) Spontaneous and DTT-induced DsRed expression in blocked by the presence of shRNA-RTA. (C) Western blot analysis of whole cell lysates of the Clone 5 cells untreated or treated with DTT with polyclonal rabbit anti-RTA. RTA is present in untreated Clone 5 cells representing the high spontaneous lytic replication seen in this cell line and is further induced by DTT treatment. β-actin acts as a loading control. (D) RT PCR amplification of ORF29a/b from the total mRNA of Clone 5 cells expressing shRNA-GFP or shRNA-RTA, untreated or treated with DTT or 3 mM Sodium Butyrate (NaBut). Lower levels of ORF29a/b transcript are seen in shRNA-RTA-expressing samples. RT PCR for β actin on the same RNA was used to normalise cDNA input.

3.2.6 A comparison of the gene expression profiles of Clone 5 and Clone 9 cells.

We have observed a greater level of spontaneous lytic reactivation in Clone 5 cells compared to Clone 9 cells (section 3.2.5). Increased levels of XBP-1s in resting cells could not account for this phenotype (section 3.2.5.2). We have also shown that, in Clone 5 and Clone 9 cells, RTA expression is necessary for lytic replication. In this section, we aimed to identify cellular genes with differential expression between Clone 9 and Clone 5 cells that could be responsible for the difference in lytic replication observed. To this end we compared the gene expression profiles of untreated Clone 9 and Clone 5 cells. To specifically determine those differential genes that were independent of increased lytic reactivation in Clone 5 cells, we also compared the gene expression profile of Clone 5 cells transduced with lentivirus encoding shRNA targeting RTA or GFP as control, at an MOI 8. We have seen in section 3.2.5.3 that the expression of shRNA-RTA effectively suppresses KSHV lytic replication in Clone 5 cells. 6 hours post-transduction the cells were washed once and resuspended in fresh medium. 24 hours later these cells were transduced a second time with lentivirus encoding shRNA targeting RTA or GFP as control at an MOI 8 and again washed 6 hours post-transduction. 72 hours post-transduction, the cells were pelleted and resuspended in TRIzol reagent. Samples of Clone 9 and Clone 5 cells non-transduced or transduced with control vectors were also resuspended in TRIzol reagent. Total RNA was purified from all samples and the quality and quantity assessed using an Agilent bioanalyzer. From this, mRNA was labelled according to the materials and methods (see section 2.11.2). Labelled cDNA was mixed with Cy3-labelled reference RNA and hybridised to Agilent Whole Human Genome Oligo Microarrays. A common reference RNA mixture was used to enable comparison across the whole sample set.

Intensities for each sample relative to the reference were obtained from the feature extraction file by background subtraction and scale normalisation using R and BioConductor (Gentleman et al., 2004). Differentially expressed genes were identified by constructing linear models using the Limma BioConductor library (Smyth, 2005). The samples were split into three groups: Clone 9 (CL9), Clone 5 (CL5), which includes Clone 5 cells expressing the control short-hairpin

GFP (CL5shGFP) and finally, the short-hairpin RTA-expressing Clone 5 cells (CL5shRTA). Fold changes for two comparisons (CL9/CL5 and CL5shRTA/CL5) were calculated and used to construct lists of significantly differentially expressed genes (Benjamini-Hochberg adjusted p-value < 0.01). Probes with mean intensities across all 10 samples less than twice the background level were removed ($1/2\log_2 RG < 7$).

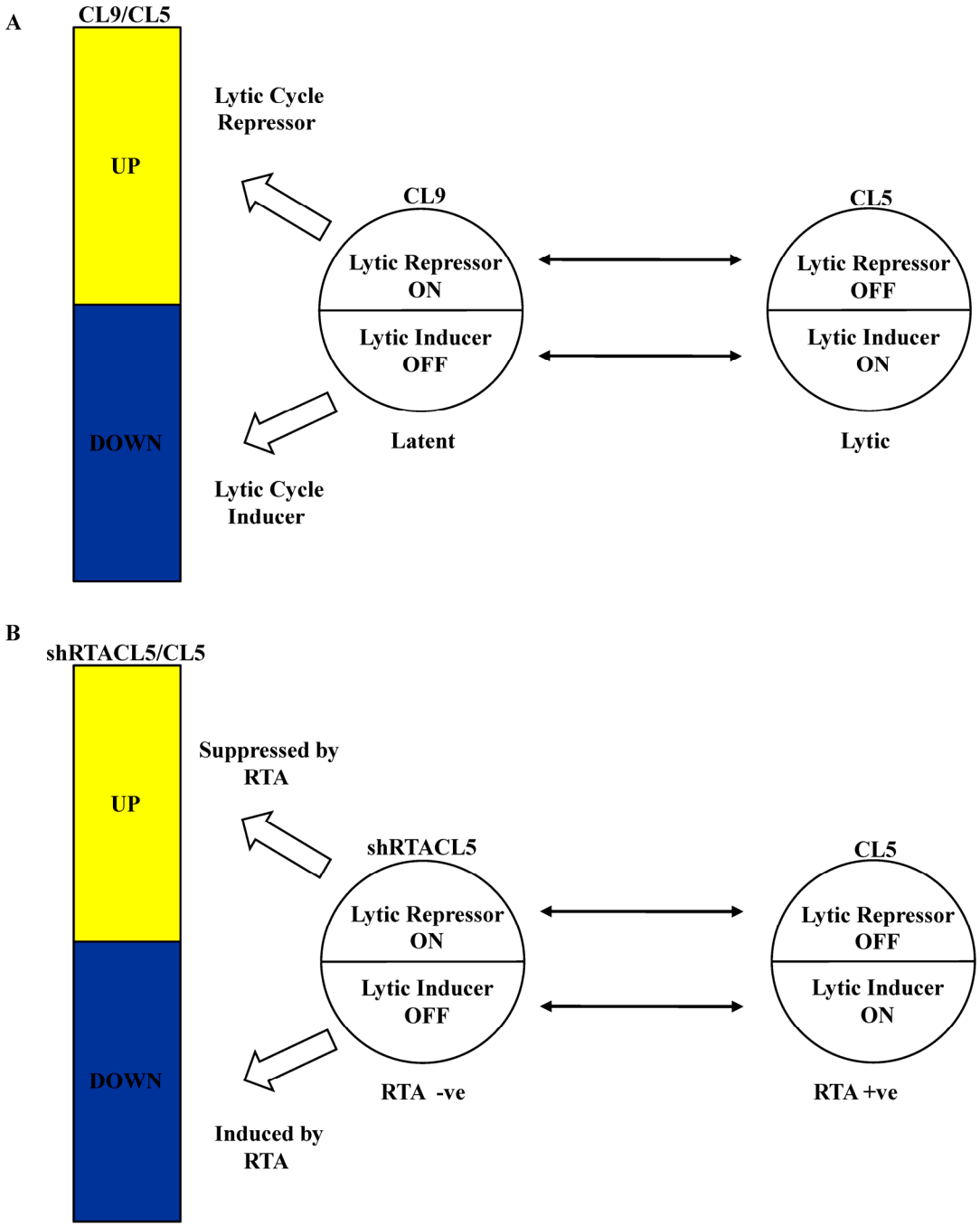


Figure 3-14 Understanding the difference between Clone 9 and Clone 5 cells
Diagrams representing the scenarios that could account for the difference in lytic replication seen between Clone 9 and Clone 5 cells. (A) Genes upregulated (yellow) in this first comparison between Clone 9 (CL9) and Clone 5 (CL5) cells represent potential repressors of KSHV lytic cycle, whereas downregulated (blue) genes are potential inducers. (B) Genes upregulated (yellow) in this first comparison between shRNA to RTA-expressing Clone 5 (shRTACL5) and

Clone 5 (CL5) cells represent those genes that are suppressed by KSHV lytic RTA whereas downregulated (blue) genes are induced by RTA.

As described earlier in the chapter, we observe less spontaneous lytic replication in Clone 9 cells than in the Clone 5 cells. Thus either Clone 9 cells express a repressor of lytic replication that is suppressed in Clone 5 cells or Clone 5 cells express an inducer of the lytic cycle that is not expressed in Clone 9 cells (Figure 3-14 A). A comparison of these two samples reveals 383 genes that are differentially expressed between Clone 9 and Clone 5 cells (CL9/CL5 list). Of these, 80 genes are upregulated (yellow) in Clone 9 compared to Clone 5 cells and could represent potential repressors of KSHV reactivation. The remaining 303 genes are downregulated (blue) in Clone 9 compared to Clone 5 cells and could represent potential inducers of KSHV reactivation (Figure 3-14 A). However, a number of these genes could have differential expression due to the presence of RTA and the KSHV lytic cycle in Clone 5 cells.

Looking directly at the effect of RTA and the lytic cycle on gene expression we compared Clone 5 cells expressing shRNA-RTA to un-transduced Clone 5 cells and Clone 5 cells expressing shRNA-GFP control (shRTACL5/CL5 list). 78 genes were upregulated (yellow) and 117 genes were downregulated (blue) in the absence of RTA (Figure 3-14 B). These differentially expressed genes are dependent on the RTA-induced lytic environment in Clone 5 cells and can therefore be regarded as false positives. Without further investigation it is not possible to determine whether the level of these genes change the replication state or are altered by the replication state itself. These genes were used to filter the genes in the CL9/CL5 list in order to obtain those genes that represent the potential inducers or repressors of KSHV lytic replication that are independent of the lytic cycle in Clone 5 cells (Figure 3-15 A).

Our analysis of the differences between the gene expression profiles of Clone 9 and Clone 5 cells led to the identification of several possible scenarios in which potential repressors or inducers could be identified (Figure 3-14). By translating these scenarios into comparisons between the samples we were able to characterise differentially expressed genes by how they may regulate RTA expression and therefore control KSHV reactivation (Figure 3-15 A and B).

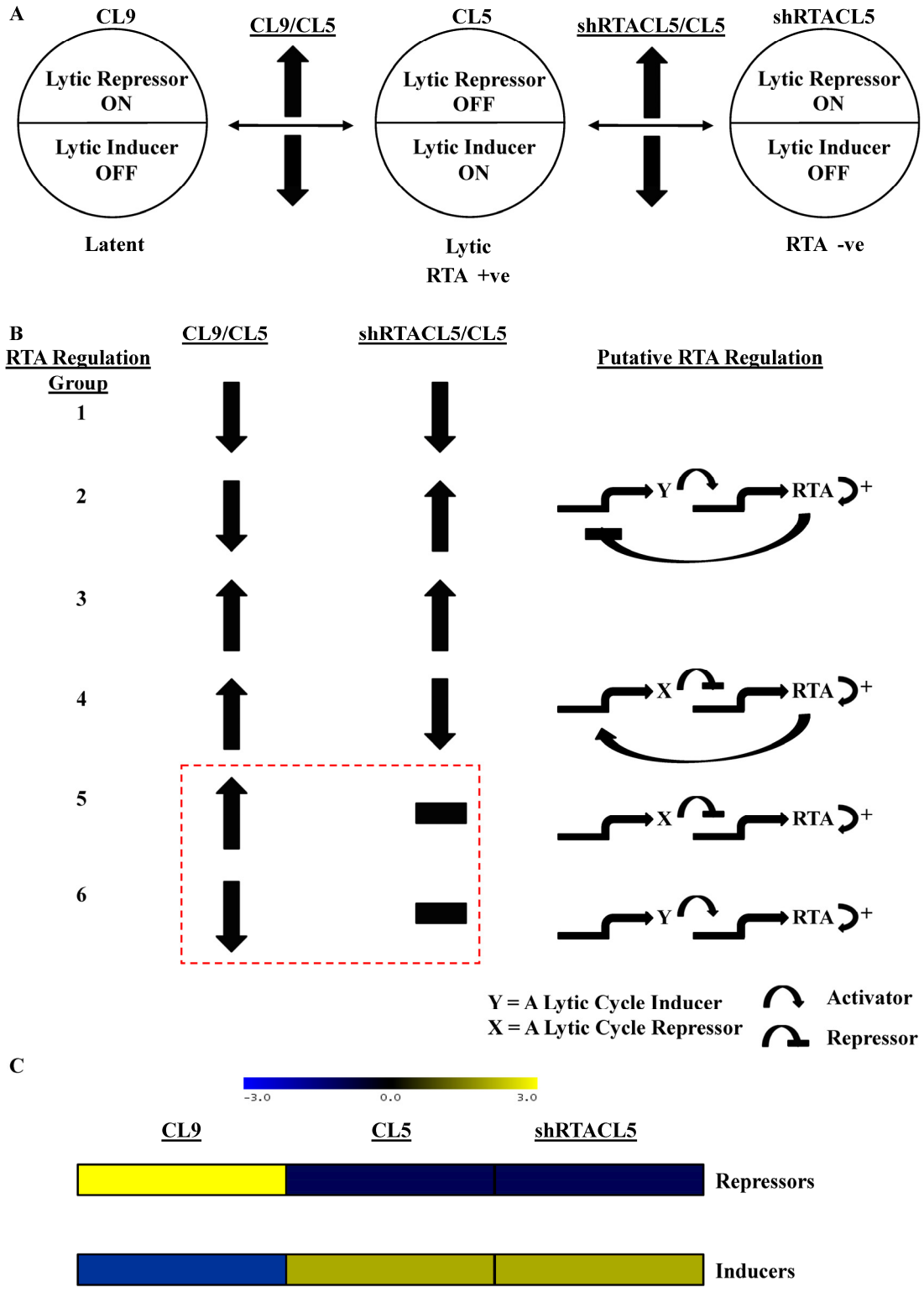


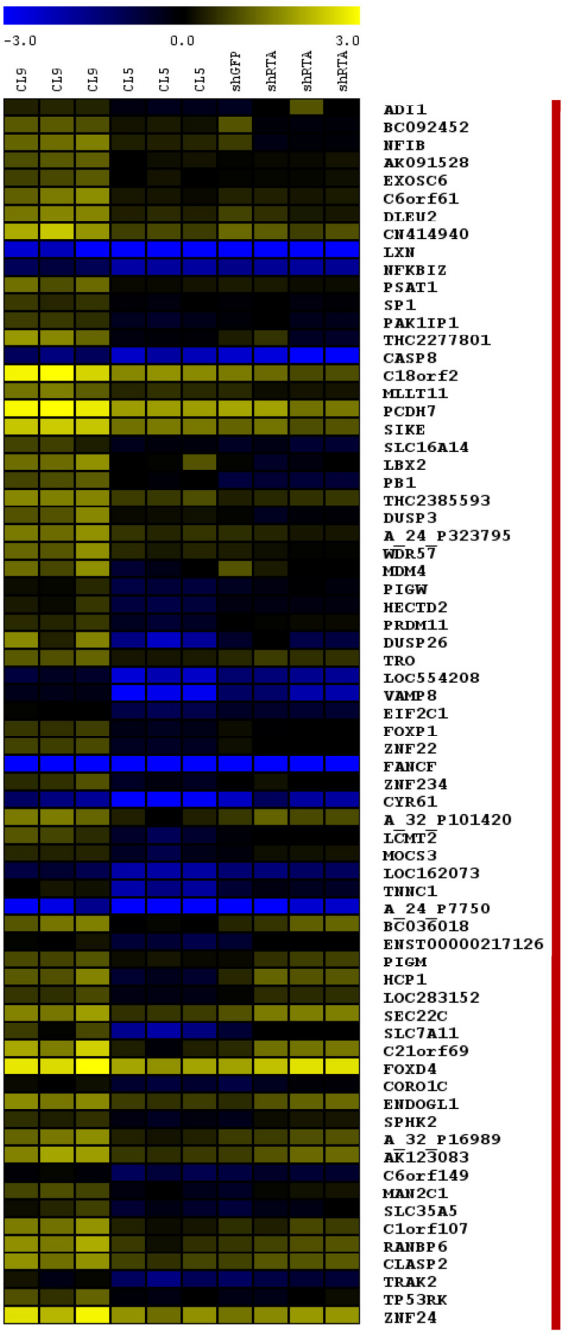
Figure 3-15 Constructing a gene list
(A) Diagrammatic representation of the comparisons being made between Clone 9 (CL9) samples to Clone (CL5) and shRNA-RTA-expressing Clone 5 cells (shRTACL5) to CL5 cells. For each comparison genes can be upregulated (↑), downregulated (↓) or neither (-). (B) Genes that are upregulated or downregulated in both comparisons RTA regulation groups 1 and 3 (↑↑ and ↓↓) or in groups 2 and 4 (opposite genes) were removed from the final gene list as their expression is dependent on RTA and lytic cycle. The final gene list is therefore composed of genes that fall into RTA regulation groups 5 and 6 (unique genes) highlighted by the red box. (C) Representations of the heatmap gene expression patterns expected based on RTA regulation group criteria. Group 5: CL9 up compared to CL5 and shRTACL5 similar to CL5, potential repressors of KSHV lytic replication and Group 6: CL9 down compared to CL5 and shRTACL5 similar to CL5, potential inducers of KSHV lytic replication.

Genes found to be upregulated in both comparisons or downregulated in both comparisons, members of RTA regulation groups 1 and 3, were removed from the analysis. These genes are dependent on RTA and KSHV lytic replication as discussed above. Those genes that fall into the RTA regulation groups 2 and 4, have opposite gene expression levels, 'opposite genes', and are also regulated by RTA expression, possibly directly, but in a complex way. For example, genes present in group 2 are the potential inducers of KSHV lytic cycle that are suppressed by RTA possibly indicating a negative feedback interaction. RTA regulation groups 5 and 6 represent 'unique genes', these genes are differentially expressed in CL9 and CL5 and are independent of RTA; as their expression is not changed in the presence of shRNA-RTA (Figure 3-15 B). Some of these cellular genes are likely potential repressors (Group 5) and inducers (Group 6) of KSHV lytic replication. Therefore genes in RTA regulation groups 5 and 6 were included in the final list of differentially expressed genes. This is outlined in Figure 3-15 B, the final gene list being constructed from the genes in red boxes.

The final filtered gene list was then split into two lists of upregulated and downregulated genes. The Ensembl BioMart server was used to convert the Agilent probe identifiers to Entrez Gene IDs using the BioConductor BioMart package (Durinck et al., 2005). These were then uploaded to the functional annotation clustering tool on the DAVID website (http://david.abcc.ncifcrf.gov/) to identify significantly over-represented functional groups within each list (Benjamini-Hochberg adjusted p-value < 0.05) (Huang da et al., 2009) (Dennis et al., 2003). The broad annotation clusters created by DAVID were kept but sub-clusters that had Benjamini-Hochberg adjusted p-values greater than 0.05 were removed, yielding 4 annotation clusters consisting of 72 genes. All of these genes were downregulated in Clone 9 compared to Clone 5 cells and represent potential inducers of the lytic cycle (Figure 3-16 B). These genes were present in one or more of the four broad functional groups, nervous system development – 27 genes, anatomical structure morphogenesis – 27 genes, cell differentiation – 35 genes and plasma membrane part – 38 genes. The 69 genes that were upregulated in Clone 9 compared to Clone 5 cells, and therefore represent potential repressors of KSHV lytic replication, did not cluster

into over-represented functional groups, therefore all genes are shown in the heatmap (Figure 3-16 A).

A



B

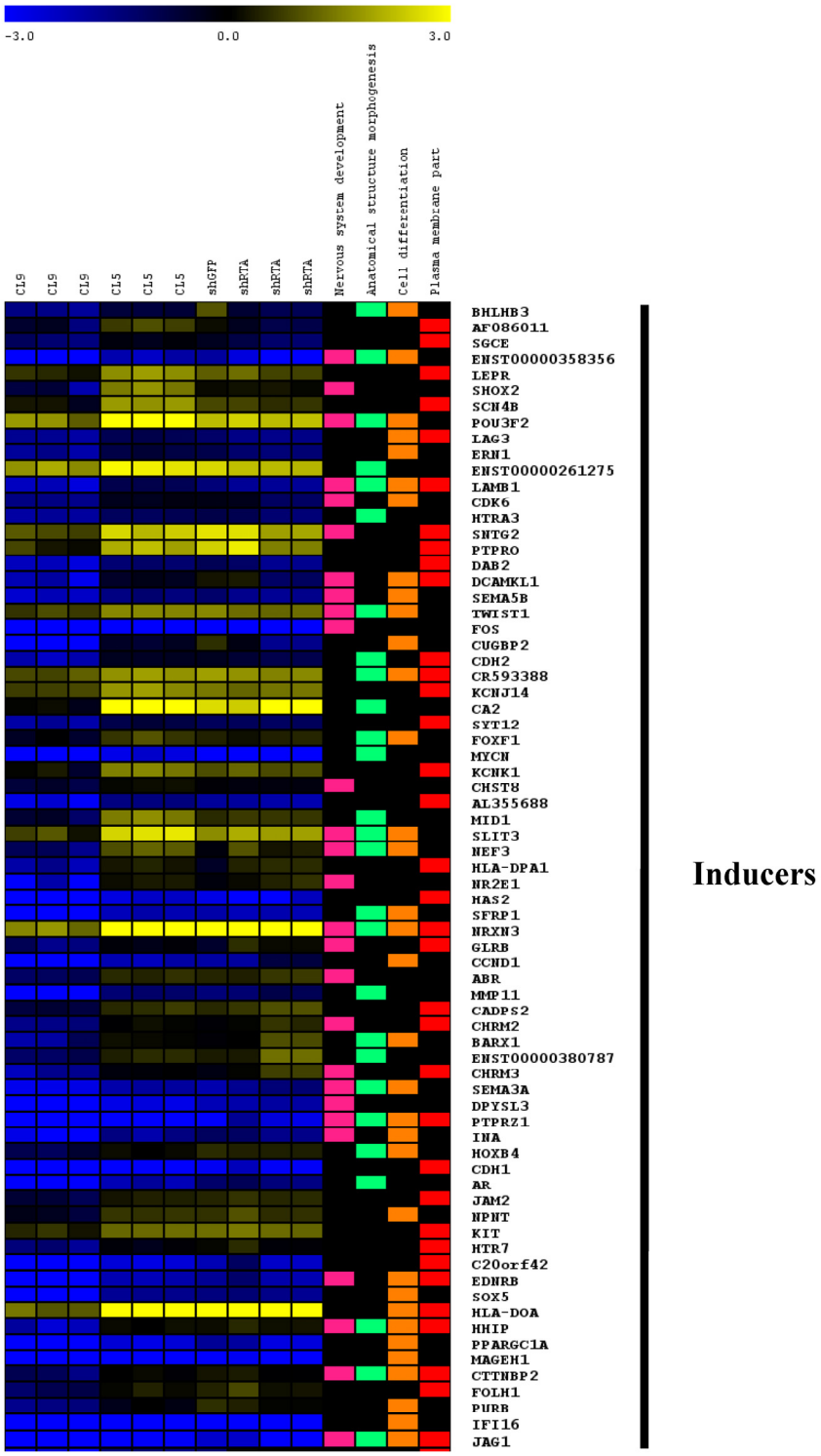


Figure 3-16 Heatmap representation of differentially expressed genes between Clone 9 and Clone 5 cells

(A) Heatmap of all genes significantly upregulated in Clone 9 compared to Clone 5 after RTA and lytic cycle dependent genes were removed – Potential repressors (B) Heatmap of significantly over-represented functional groups within the downregulated gene lists after RTA and lytic cycle dependent genes were removed – Potential inducers. Each column represents one sample and each row one gene. Gene expression is shown as a pseudo-coloured representation of log(2) expression ratio with yellow being above and blue below the row/column median level of expression (set to 0) as shown by the scale. DAVID was used to annotate the downregulated genes into broad functional groups colour coded to right of heatmap.

From the potential repressors and inducers identified we could then select, using the heatmap (Figure 3-16), those genes that best represent the expression criteria set out by the RTA regulation groups (Figure 3-15 C) and focus on these genes as potential regulators of KSHV lytic replication (Figure 3-17). The genes that could potentially be involved in the repression of KSHV lytic cycle and most closely fit the criteria of RTA regulation group 5 are given in the table A while potential inducers are listed in the table B (see Figure 3-17).

A

Potential Repressors	
NFIB	Homo sapiens nuclear factor I/B (NFIB), mRNA [NM_005596]
NFKBIZ	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta [NM_031419]
PSAT1	Homo sapiens phosphoserine aminotransferase 1 (PSAT1), transcript variant 1, mRNA [NM_058179]
SP1	HIomo sapiens Sp1 transcription factor (SP1), mRNA [NM_138473]
PAK1P1	Homo sapiens PAK1 interacting protein 1 (PAK1IP1), mRNA [NM_017906]
CASP8	Homo sapiens caspase 8, apoptosis-related cysteine protease [NM_033356]
SLC16A14	Homo sapiens solute carrier family 16 (monocarboxylic acid transporters), member 14 (SLC16A14), mRNA [NM_152527]
DUSP3	Homo sapiens dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related) (DUSP3), mRNA [NM_004090]
DUSP26	Homo sapiens dual specificity phosphatase 26 (putative) (DUSP26), mRNA [NM_024025]
EIF2C1	Homo sapiens eukaryotic translation initiation factor 2C, 1 (EIF2C1), mRNA [NM_012199]
FOXP1	Homo sapiens forkhead box P1 (FOXP1), transcript variant 1, mRNA [NM_032682]
ZNF22	Homo sapiens zinc finger protein 22 (KOX 15) (ZNF22), mRNA [NM_006963]
ZNF234	Homo sapiens zinc finger protein 234 (ZNF234), mRNA [NM_006630]
SLC35A5	Homo sapiens solute carrier family 35, member A5 (SLC35A5), mRNA [NM_017945]
RANBP6	Homo sapiens RAN binding protein 6 (RANBP6), mRNA [NM_012416]
CLASP2	Homo sapiens cytoplasmic linker associated protein 2 (CLASP2), mRNA [NM_015097]
TP53RK	Homo sapiens TP53 regulating kinase (TP53RK), mRNA [NM_033550]

B

Potential Inducers	
PTPRO	Homo sapiens protein tyrosine phosphatase, receptor type, O [NM_002848]
CA2	Homo sapiens carbonic anhydrase II [NM_000067]
FOXF1	Homo sapiens forkhead box F1 (FOXF1), mRNA [NM_001451]
KCNK1	Homo sapiens potassium channel, subfamily K, member 1 [NM_002245]
NEF3	Homo sapiens neurofilament 3 (150kDa medium) (NEF3), mRNA [NM_005382]
HLA-DPA1	Homo sapiens major histocompatibility complex, class II, DP alpha 1 (HLA-DPA1), mRNA [NM_033554]
ABR	Homo sapiens active BCR-related gene (ABR), transcript variant 1, mRNA [NM_021962]
CADSP2	Homo sapiens Ca2+-dependent activator protein for secretion 2 (CADPS2), transcript variant 1, mRNA [NM_017954]
CHRM2	Homo sapiens cholinergic receptor, muscarinic 2 (CHRM2), transcript variant 1, mRNA [NM_001006630]
BARX1	Homo sapiens BarH-like homeobox 1 (BARX1), mRNA [NM_021570]
CHRM3	Homo sapiens cholinergic receptor, muscarinic 3 (CHRM3), mRNA [NM_000740]
HOXB4	Homo sapiens homeobox B4 (HOXB4), mRNA [NM_024015]
JAM2	Homo sapiens junctional adhesion molecule 2 (JAM2), mRNA [NM_021219]
NPNT	Homo sapiens nephronectin (NPNT), mRNA [NM_001033047]
HHIP	Homo sapiens hedgehog interacting protein (HHIP), mRNA [NM_022475]
CTTNBP2	Homo sapiens cortactin binding protein 2 (CTTNBP2), mRNA [NM_033427]
FOLH1	Homo sapiens folate hydrolase (prostate-specific membrane antigen) 1 (FOLH1), transcript variant 1, mRNA [NM_004476]

Figure 3-17 Table of potential repressors and inducers of KSHV reactivation
Genes that most obviously satisfy the individual regulation group criteria were selected. (A) Potential repressors of KSHV lytic replication are upregulated in Clone 9 compared to Clone 5

cells and are not affected by RTA expression. (B) Potential inducers of KSHV lytic replication. All are downregulated in Clone 9 compared to Clone 5 cells and are not affected by RTA expression.

Of those genes identified as potential repressors several candidates are worthy of selection for further investigation based on previous studies (Figure 3-17 Table A). Candidates include two genes that are involved in the regulation of the NF-κB pathway. This pathway is activated in latently infected cells by the KSHV v-FLIP protein (see section 1.1.12.4). When PEL cells were treated with Bay11-7082, a pharmacological inhibitor of NF-κB, increased KSHV lytic replication was seen, indicating a role for the NF-κB pathway in maintaining latency (Brown et al., 2003). NF-κB has also been shown to negatively regulate RTA expression by antagonising the coactivator RBP-Jk (Izumiya et al., 2009). However NF-κB is highly active in cells undergoing lytic replication and therefore if p65 does act to inhibit reactivation then this must be overcome during the lytic cycle. Indeed the dependence on NF-κB activity for KSHV lytic replication is cell type dependent, with primary infected endothelial cells behaving like PEL cells, but infected human fibroblasts showing no increase in KSHV reactivation when the NF-κB pathway is inhibited (Grossmann and Ganem, 2008).

The two genes highlighted by this microarray study as potential KSHV lytic cycle repressors are the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFKBIZ) and the transcription repressor, forkhead box P1 (FOXP1). The full-length form of FOXP1 has been shown to inhibit the transcription of NF-κB responsive genes; however truncated forms of the protein have been associated with NF-κB activity in some DLCBLs and follicular lymphomas (FLs) (Green et al., 2009). This suggests that truncated forms of FOXP1 act as dominant negatives, inhibiting the function of FOXP1 and allow NF-κB to function. Further study would require identification of the isoform expressed in Clone 9 cells and its effect on NF-κB activity. If a truncated form is found then its introduction into Clone 5 cells should show an increase in NF-κB activity and a decrease in the lytic replication observed. It would also be interesting to ascertain which form of FOXP-1 is present in PEL cells and whether this contributes to the increased NF-κB activity seen.

NFKBIZ is a known inhibitor of the NF-κB pathway therefore its contribution to the repression of KSHV lytic cycle is unclear. However, in the context of the infected cell environment with increased NF-κB activity through vFLIP, a complex mechanism of regulation is expected and may require inhibitors to balance the pathway (Yamazaki et al., 2001). In fact NF-κB is known to induce its own inhibitors leading to a negative feedback mechanism of regulation (Brown et al., 1993). Further investigation is required in order to determine the exact role of NFKBIZ in KSHV reactivation possibly via regulation of the NF-κB pathway.

Another of the genes highlighted as a potential repressor of KSHV lytic replication and independent of RTA expression is the human Argonaute protein, eukaryotic translation initiation factor 2C, 1 (EIF2C1/hAgo2) (reviewed in (Carmell et al., 2002)). This protein is required for short interfering RNA mediated post-transcriptional gene silencing in mammalian cells (Doi et al., 2003). Recent studies have identified several KSHV microRNAs that are involved in providing fine control over KSHV reactivation (Bellare and Ganem, 2009) (Lei et al., 2010) (Lu et al., 2010) (reviewed in section 1.1.12.7). The functions of these viral microRNAs rely on the same machinery as the host cellular miRNA and therefore any perturbations in this pathway may affect the control of KSHV lytic replication. Further investigation into the role of EIF2C1 in maintaining KSHV latency through the processing of KSHV miRNAs is required but is technically difficult.

Finally two dual specific phosphatases, DUSP3 and DUSP26 were identified as potential repressors of KSHV reactivation by the microarray analysis. The proteins encoded by these genes negatively regulate members of the mitogen-activated protein (MAP) kinase superfamily (MAPK/ERK, SAPK/JNK, p38), by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues. These proteins have distinct substrate specificities for the various MAP kinases and have different tissue distribution and subcellular localization. DUSP3 inhibits JNK activity while DUSP26 can inactivate p38 (Arnoldussen et al., 2008) (Yu et al., 2007c). Recently the MAPKs, MEK/ERK, JNK and p38 have been shown to have a role in KSHV primary infection as well as in reactivation (Pan et al., 2006) (Cohen et al., 2006) (Xie et al., 2008) (Ford et al.,

2006). In response to TPA, ERK and p38 activity is enhanced and induces KSHV reactivation via the transcriptional activator AP-1 which binds the RTA promoter directly. Inhibition of ERK, JNK and p38 pathways resulted in reduced TPA-induced reactivation (Xie et al., 2008). The reduced expression of a negative regulator of MAPK activity, such as the DUSPs, may therefore lead to the increased spontaneous reactivation seen in Clone 5 cells. Further study to confirm this hypothesis is required, including overexpression of these proteins in Clone 5 cells, which should reduce the level of DsRed seen in resting cells compared to control.

Of the genes identified as potential inducers of KSHV lytic replication only cyclin D1 (CCND1) could be selected based on previous studies, for further investigation, (Figure 3-17 B). The G1-S phase transition of the cell cycle is controlled by cyclin dependent kinases 4 and 6 (cdks) in combination with D-type cyclins. DNA is replicated during this stage of the cell cycle including KSHV progeny virus DNA and viral DNA tethered as an episome to the host chromosome. Recently a study using TPA as an inducer of KSHV lytic replication reported a 30 % increase in virus production when BCBL-1 cells were synchronised and TPA-induced during S-phase (McAllister et al., 2005). KSHV encodes a homologue of cellular cyclin D, v-cyc, which is thought to be important in driving cell proliferation but may also be important in preparing the cell for viral DNA replication during lytic reactivation (Bryan et al., 2006) (see section 1.1.12.3). Therefore deregulated cyclin D expression in Clone 5 cells may lead to a larger number of the cells in the S-phase of the cell-cycle, with a concomitant increase in spontaneous lytic replication. In order to further investigate this hypothesis, it would be important to look at the cell cycle profile of Clone 5 cells as well as determine the effect of overexpressing cellular cyclin D on the level of lytic reactivation in Clone 9 cells.

3.3 Discussion

KSHV has been shown to infect more than 20 cell types *in vitro*, predominantly establishing latent infection (Renne et al., 1998) (Blackbourn et al., 2000b) (Bechtel et al., 2003). KSHV infected cell lines, therefore, provide valuable tools for the study of factors that can induce the switch between latent and lytic phases of the life cycle. However, until recently, the assays available for monitoring KSHV lytic replication were limited to measuring virus production and the detection of viral gene products by PCR or western blotting. These techniques require destruction of cell cultures and/or vast amounts of processing, delaying any investigation into KSHV reactivation. It is also difficult to observe subtle changes in reactivation levels using these assays because the entire population is assessed. Therefore, any small but physiologically relevant changes may be masked by the spontaneous reactivation levels present or diluted out by the sheer number of cells. In this chapter we have developed and characterised a system based on the Vieira Laboratory's recombinant virus, rKSHV.219, to facilitate further investigation into the cellular regulators of KSHV reactivation (Figure 3-1). Using fluorescent markers, the rKSHV.219 system allows the rapid identification of latently infected cells by GFP expression. Initiation of lytic replication in these cells is further indicated by the co-expression of DsRed fluorescence.

Before using this system to reveal potential activators of KSHV lytic replication we tested the response of rKSHV.219 infected HEK-293T cells to known inducers of KSHV reactivation (Figure 3-2, Figure 3-3 and Figure 3-11). The response of Clone 9 to the phorbol ester, TPA, was weak but has been seen in other studies (Bechtel et al., 2003) (Vieira and O'Hearn, 2004). It has also been reported that cells synchronised in S-phase of the cell cycle respond better to TPA-induced reactivation (McAllister et al., 2005). However, the lytic cycle was stimulated in response to all other inducers; confirming that the rKSHV.219 infected HEK-293T cell lines generated did represent authentic KSHV infected cell lines.

While carrying out these experiments it was evident that, in the same sample, the level of DsRed expression seen by microscopy was consistently greater

than that detected by FACsArray. The data generated by FACsArray did provide the same conclusions as the microscope i.e. the number of DsRed-positive cells increase on induction of KSHV reactivation, but the percentages given each time were an underestimate. On further investigation we discovered that the FACsArray, which does not detect GFP fluorescence essential for this system, was unable to detect all cells expressing DsRed. DsRed has a maximal absorbance of 561 nm and maximal emission range from 525 - 675 nm. The FACsArray has a filter which can detect wavelengths between 564 - 606 nm, while the excitation wavelength was set at 532 nm. This was therefore not an optimal set up for the detection of cells expressing varying levels of DsRed. The confocal microscope however was set at an excitation wavelength of 568 nm and was therefore able to excite and visualise a greater number of DsRed-expressing cells. Direct comparison showed that the microscope detected at least 3-fold more DsRed-expressing cells than the FACsArray (Figure 3-3 D). Despite this we have demonstrated that the rKSHV.219 infected HEK 293T cells act as a robust sensor for KSHV lytic replication, and can be used for the study of physiological inducers of reactivation.

The rKSHV.219 system's ability to report KSHV lytic reactivation is reliant on DsRed expression driven by the PAN lytic promoter. Studies by Vieira *et.al.* have shown that ectopic expression of RTA leads to DsRed expression and infectious virus production in rKSHV.219 infected cell lines, and that all DsRed-positive cells express lytic antigens (Vieira and O'Hearn, 2004). To ensure that the DsRed fluorescent expression we saw in the rKSHV.219 HEK 293T cell lines was exclusively caused by the presence of RTA we used RNA interference. Expression of shRNA to RTA, but not GFP, in rKSHV.219 HEK 293Ts reduced DsRed expression as well as the expression of the lytic transcript ORF29a/b in response to known inducers of KSHV reactivation (Figure 3-4 and Figure 3-13). We were therefore confident that the rKSHV.219 HEK 293T cell systems established respond, as expected, to challenges known to induce KSHV lytic cycle and that DsRed fluorescence was directly dependent on RTA expression.

By creating and characterising a range of clonal cell lines, derived from the rKSHV.219 infection of HEK-293T cells, we hoped to use the system to isolate

additional cellular processes involved in KSHV reactivation. DsRed expression in these cells results from three rounds of signal amplification, first the direct activation of RTA by the cellular gene, secondly the auto-activation of the RTA promoter by RTA itself and finally RTA activation of the PAN promoter. This renders the rKSHV.219 reporter system highly sensitive to KSHV lytic replication. The difference in DsRed expression seen between clonal cell lines, for example between Clone 5 and Clone 9 cells, is therefore significant and can be investigated. By microarray analysis we hoped to explain the difference in the degree of spontaneous reactivation occurring in these two cell lines (see section 3.2.6). This analysis provided several candidates for potential inducers and repressors of KSHV lytic replication however, further investigation is required to confirm these associations. This work shows that the rKSHV.219 system is not only valuable as a reporter system but has the potential to identify novel virus-host interactions when different phenotypes are observed.

Using the characterised rKSHV.219 HEK 293T cell, Clone 9, system we also provided further evidence that KSHV lytic replication is initiated by the cellular stress induced basic leucine zipper transcription factor, XBP-1s (Wilson et al., 2007). By RNA interference we were able to inhibit the ability of endogenous XBP-1s to induce reactivation (Figure 3-7). Recent studies that have employed genome-wide screens in an attempt to systematically identify cellular signals involved in KSHV reactivation have also revealed XBP-1 (Yu et al., 2007b) (Bryan et al., 2006). XBP-1s is an important mediator of the unfolded protein response (UPR) and is activated by several physiological conditions including B-cell receptor ligation and importantly hypoxia investigated in chapter 4 (Yan et al., 2008) (Skalet et al., 2005) (Thuerauf et al., 2006) (Feldman et al., 2005) (Koumenis and Wouters, 2006). XBP-1 is also a necessary transcription factor for the terminal differentiation of B-cells to plasma cells (Reimold et al., 2001) (Iwakoshi et al., 2003).

PEL cells are latently infected with KSHV and phenotypically resemble plasmablast cells, a B-cell stage prior to antibody-secreting plasma cell (Jenner et al., 2003) (Klein et al., 2003) (Tarte et al., 2003). KSHV infected PEL cells predominantly express XBP-1u but when XBP-1s is exogenously supplied the KSHV lytic cycle is activated (Wilson et al., 2007) (Yu et al., 2007b). In addition,

the morphology and gene expression profiles of these cells reveals a phenotype that closely resembles plasma cells, investigated further in chapter 5 (Wilson et al., 2007) (Shaffer et al., 2004). This data and the evidence provided in chapter 3 suggest that plasma cell differentiation and XBP-1s expression can act as a cellular cue for the induction of the KSHV lytic cycle. By preparing the infected B-cell for protein production, XBP-1s provides an ideal environment for lytic replication. This raises the possibility that in KSHV infected B-cells latency is maintained until plasma cell differentiation; which through XBP-1s causes reactivation of KSHV. If this occurs in the reticulated epithelium of the oral cavity and the lymphoid tissue of the Waldeyers ring, KSHV could be shed from infected plasma cells along with antibodies into the saliva (Cattani et al., 1999) (Koelle et al., 1997). KSHV reactivation from latency in B-cells is probably responsible for initiating the waves of transient viraemia reported in the saliva of KSHV positive individuals, and is important in viral transmission (Pauk et al., 2000).

Understanding whether XBP-1s directly transactivates the ORF50 promoter or activation results from indirect interactions or downstream effects of XBP-1s overexpression, is central to understanding the role of XBP-1s in KSHV reactivation. We were able to show that the mechanism for XBP-1s induced reactivation is direct transactivation of the ORF50 promoter via a XBP-1s responsive element ‘ACGT’ (Figure 3-8). This reporter experiment was carried out in virus-free HEK 293T cells therefore, due to absence of endogenous RTA, auto-activation of the ORF50 promoter is not seen (Deng et al., 2000). The ability of XBP-1s to induce RTA expression is still observed but this activity would be enhanced in the presence of endogenous RTA. The precise mechanism of XBP-1s transactivation of the ORF50 promoter is still not fully clear, as it is not yet known whether XBP-1 binds as a homodimer or as a heterodimer with another bZIP containing partner for example ATF-6 (Newman and Keating, 2003). It is also not known how plasma cell differentiation affects the epigenetic modifications that are known to influence the switch from latency to lytic replication, for example ORF50 promoter demethylation (Chen et al., 2001) (Gwack et al., 2001) (Lu et al., 2010) (reviewed in (Pantry and Medveczky, 2009)).

A recent study demonstrated the ability of mouse XBP-1s to act on the KSHV RTA promoter (Yu et al., 2007a). This not only supports our findings but also indicates that XBP-1 and the response elements it recognises are potentially conserved amongst species. In fact alignment of the human and mouse XBP-1 DNA sequences reveals 77 % similarity (data not shown). It has also recently been observed that XBP-1s is associated with reactivation of EBV, the most closely related human virus to KSHV (Sun and Thorley-Lawson, 2007) (Bhende et al., 2007) (McDonald et al., 2010). We therefore reasoned that the mechanism of XBP-1s reactivation may be exploited by other viruses in the same genus as KSHV. To address this hypothesis we carried out ORF50 promoter analysis for potential XBP-1 binding elements, indentified by Acosta-Alvear, on several Rhadinoviruses (Acosta-Alvear et al., 2007). The RTA/ORF50 proteins of gamma-2-herpesviruses have been shown to have significant homology and the ability to act on each other's promoters (Damania et al., 2004), providing further evidence that methods for ORF50 transactivation may be conserved. We also used this information to identify the accurate promoter regions for each virus studied (Figure 3-9). Once the first exon and the starting ATG was determined for each Rhadinovirus selected, we scanned the preceding 500 bp for any of the core sequences shown to be recognised by XBP-1s in previous ChIP analysis (Acosta-Alvear et al., 2007). This examination resulted in the potential consensus sequence '(A/C/T), C, G, (C/T/A)' for XBP-1 binding and transactivation of the ORF50 promoter (Figure 3-10). To confirm the functionality of the elements identified, XBP-1 from each infected species would need to be cloned and tested on the respective virus ORF50 promoter region, cloned upstream of a reporter such as luciferase (see Figure 3-8). If confirmed this would indicate that the plasma cell differentiation induced reactivation strategy may be employed by other B-cell tropic gamma-herpesviruses.

Other physiological triggers of the switch from latency to lytic phases have since been identified including activation of the Toll-like receptors (TLRs) (see section 1.1.14). Specifically, TLR 7/8 have been shown to induce KSHV lytic replication in PEL cells, linking innate immunity to reactivation (Gregory et al., 2009). This mechanism can be translated into a physiological scenario in which a B-cell latently infected with KSHV encounters a secondary pathogen that activates

TLR 7/8 and induces KSHV reactivation. Another physiological stimulus of KSHV reactivation was identified by a gain-of-function kinome screen. Pim-1 and 3 kinases were shown to phosphorylate LANA, negating its repressive effect on lytic replication (Varjosalo et al., 2008) (Cheng et al., 2009b) (Garber et al., 2002) (Lan et al., 2004). These kinases are shown to be upregulated by the cytokines present in the microenvironment of KSHV associated tumours (Bachmann and Moroy, 2005) (Ensoli et al., 2001b). Some of these cytokines, for example interferon-γ (IFNγ), have been shown to induce KSHV reactivation; possibly identifying a method of cytokine induced reactivation (Chang et al., 2000). Another relevant inducer of KSHV reactivation is the physiological stress hypoxia (Davis et al., 2001). In fact the response element identified in this chapter as XBP-1 responsive was previously identified as the hypoxia response element (HRE), HRE4 (Cai et al., 2006a). We therefore explore the mechanism of action and the physiological significance of hypoxia in KSHV reactivation further in chapter 4.

It is clear than many cellular processes regulate KSHV reactivation, and many more are yet to be discovered. Information on how every process acts individually and in combination to balance latency and lytic replication; aids are understanding of virus-host interactions and continues to emphasise how diverse and complex they can be. To expand this knowledge further in depth and systems biology based study involving genome screening techniques is required. This will be made easier by the development of model systems such as the rKSHV.219 virus for monitoring reactivation.

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4 The role of HIF-1α and XBP-1s in hypoxia-induced reactivation

4.1 Introduction

Hypoxia, acting through the transcription factor hypoxia inducible factor -1 alpha (HIF-1α) is a cellular stress demonstrated to induce the KSHV lytic cycle (Davis et al., 2001) (see section 1.5). Under normoxic conditions the HIF-α subunits are constitutively expressed but rapidly degraded (Huang et al., 1998) (Salceda and Caro, 1997). Under hypoxia the HIF-1α subunits are stabilised, accumulate and dimerise with the HIF-1β subunit (Jiang et al., 1996a). The basic helix-loop-helix transcription factor, HIF-1α acts on promoters via hypoxic response elements (HREs) to upregulate genes involved in angiogenesis and anaerobic glycolysis (Wang et al., 1995) (see section 1.5.1). The hypoxia response is therefore crucial for solid tumour cell survival (Harris, 2002). ORF50 is hypoxia inducible, with its promoter containing seven putative HREs (Haque et al., 2003), of which three respond to HIF-1α (Cai et al., 2006a). Hypoxia-induced HIF-1α is therefore a relevant biological activator of KSHV lytic replication. Reportedly, however, HIF-1α is stabilised and sequestered to the nucleus in all PEL cells although, importantly, no lytic replication is induced (Cai et al., 2006a).

HIF-1α is also important in normal mammalian physiology with a particular role in normal B-cell physiology. Indicative of hypoxia, HIF-1α is stabilised in the microenvironments of the germinal centres (GC), other secondary lymphoid tissues and bone marrow, raising the possibility that KSHV infected B-cells encountering these environments may induce viral lytic replication (Piovan et al., 2007) (Parmar et al., 2007). HIF-1α is important in normal B-cell physiology; HIF-1α knock out RAG2-/- chimeric mice display an increase in appearance of abnormal B-1-like peritoneal lymphocytes with an associated increase in the level of autoimmunity and a disturbed maturation of the B-2 cell subset in the bone marrow (Kojima et al., 2002). Concomitantly, XBP-1 alongside its function in normal B-cell development has also been shown to be necessary for tumour growth (Romero-Ramirez et al., 2004) and is activated under hypoxia (Thuerauf

et al., 2006). Together, therefore, XBP-1 and HIF-1α are both lytic cycle triggers for KSHV, with these two pathways intersecting at normal B-cell development, the response to cellular stress and promotion of tumour survival.

In chapter 3 we identified a novel and active binding site for XBP-1 in the ORF50 promoter containing the core sequence 'ACGT'; intriguingly this sequence is identical to the core sequence of HIF-1α binding HREs (Semenza et al., 1996). This exact response element was previously identified as the hypoxia response element (HRE), HRE4 in the ORF50 promoter (Cai et al., 2006a). In this chapter, due to the presence of both stress inducible factors under hypoxia and their involvement in normal B-cell biology, we investigate further their roles in KSHV reactivation in response to hypoxia.

4.2 Results

4.2.1 Establishing hypoxic culture conditions

In order to determine the involvement of the two transcription factors HIF-1α and XBP-1s in hypoxia-induced KSHV reactivation, it was essential to establish hypoxic tissue culture conditions. Cells are cultured normally prior to the experiment (see section 2.1) and on the first day of the experiment split to a density (60-70 % confluent) that will allow them to remain viable up to 72 hours. Experimental cultures are set up in parallel; one is maintained at 21 % atmospheric oxygen (normoxia) and 5 % carbon dioxide (normoxia), in a standard humidified incubator. The second culture is sealed inside an air tight container (hypoxia chamber) and flushed by gassing with a mixture of 5 % CO_2 and 95 % N_2 mix (hypoxia) (Figure 4-1 A, section 2.1). Oxygen levels in the culture medium were found consistently to be between 1-3 % using a FOXY-R stainless steel 1/16" OD Fibre Optic Probe (Ocean Optics).

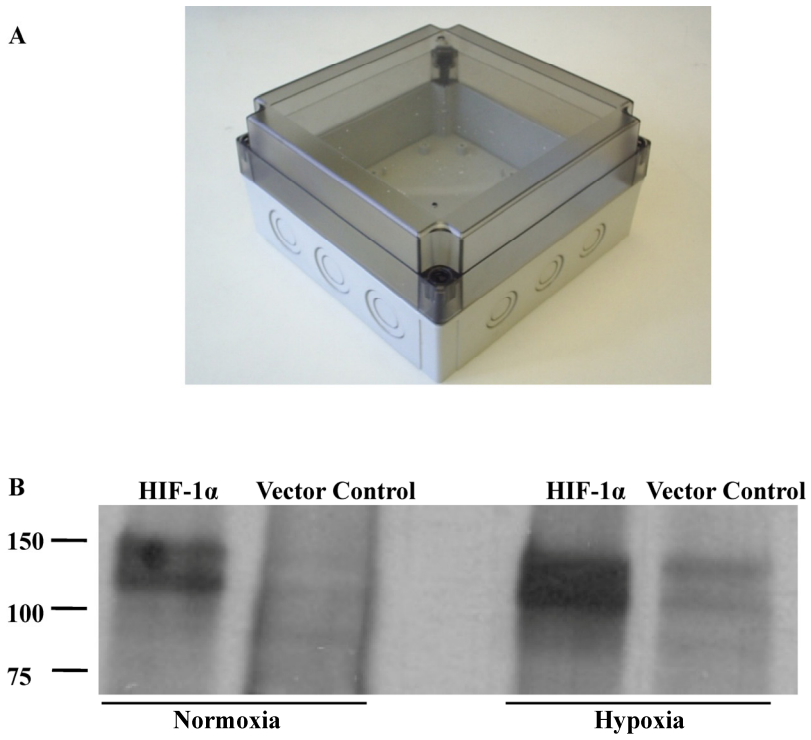


Figure 4-1 Establishing hypoxia culture conditions
(A) Hypoxic culture conditions were obtained by placing cultures in the air-tight container shown and flushing the container with 5 % CO_2 and 95 % N_2. The container is then sealed and placed within a humidified incubator. (B) Western blot analysis with anti-HIF-1α antibody (BD Transduction Labs) of whole cell lysates of HEK 293T cells transfected with pCDNA3-HIF-1α (HIF-1α lane) or empty control (vector control lane) cultured in normoxia (Normoxia; 21 %O_2) or exposed to 48 hours of hypoxia (Hypoxia; <3 % O_2). HIF-1α is expressed in pCDNA3- HIF-1α transfected samples and endogenous HIF-1α is stabilised only under hypoxic cultures relative to normoxic cultures.

To confirm that the conditions established are indeed hypoxic, we monitored the stabilisation of HIF-1α in HEK 293T cells by western blot. HIF-1α is accumulated and readily detected in HEK 293T cells transfected with empty vector and cultured under these hypoxic conditions at 48 hours, compared to normoxic controls (Figure 4-1 B). HIF-1α is also detected in HEK 293T cells transfected with a plasmid expressing HIF-1α under normoxia. Overexpression of HIF-1α under hypoxic conditions leads to a further accumulation due to the presence of endogenous HIF-1α (Figure 4-1 B). These data show that we are able to achieve sustained hypoxic culture conditions and these are sufficient to cause HIF-1α stabilisation.

4.2.2 The rKSHV.219 HEK 293T cell model and hypoxia-induced reactivation

4.2.2.1 Hypoxic suppresses DsRed expression in rKSHV.219 infected HEK 293T cells – Clone 9

Using the rKSHV.219 HEK 293T cell model for reactivation, previously established in chapter 3, we determined the level of KSHV reactivation by monitoring DsRed expression in response to hypoxic conditions. 48 hours hypoxia treatment was unable to induce DsRed expression from the PAN promoter in Clone 9 cells, compared to normoxic controls (data not shown). Hypoxia also did not induce DsRed expression in Clone 9 cells transfected with empty vector control, pCDNA3, (Figure 4-2 A and E). In addition we observed a reduction in the number of cells expressing DsRed after NaBut treatment (a stimulus known to cause reactivation; see section 3.2.2.1) under hypoxic conditions compared to normoxia (Figure 4-2 B and F). Transfection of Clone 9 cells with the RTA-expressing plasmid, pCMV-RTA, resulted in a large proportion of the cells expressing DsRed under normoxia; however, under hypoxia the level of DsRed expression and the number of DsRed-positive RTA-expressing cells were markedly reduced (Figure 4-2 C and G). Overexpression of HIF-1α in Clone 9 cells under normoxic conditions induced DsRed expression in a number of the cells; however, under hypoxia DsRed expression again was evidently reduced in these cells (Figure 4-2 D and H). We also determined DsRed expression by FACs analysis. It is clear that the number of cells expressing DsRed detected by the FACsArray does not correspond to the

number of DsRed-positive cells seen by the microscope (Figure 4-2), explained in chapter 3. However, the patterns of expression of DsRed reported by the FACsArray followed the same trend whereby hypoxia decreases DsRed expression even when RTA is expressed.

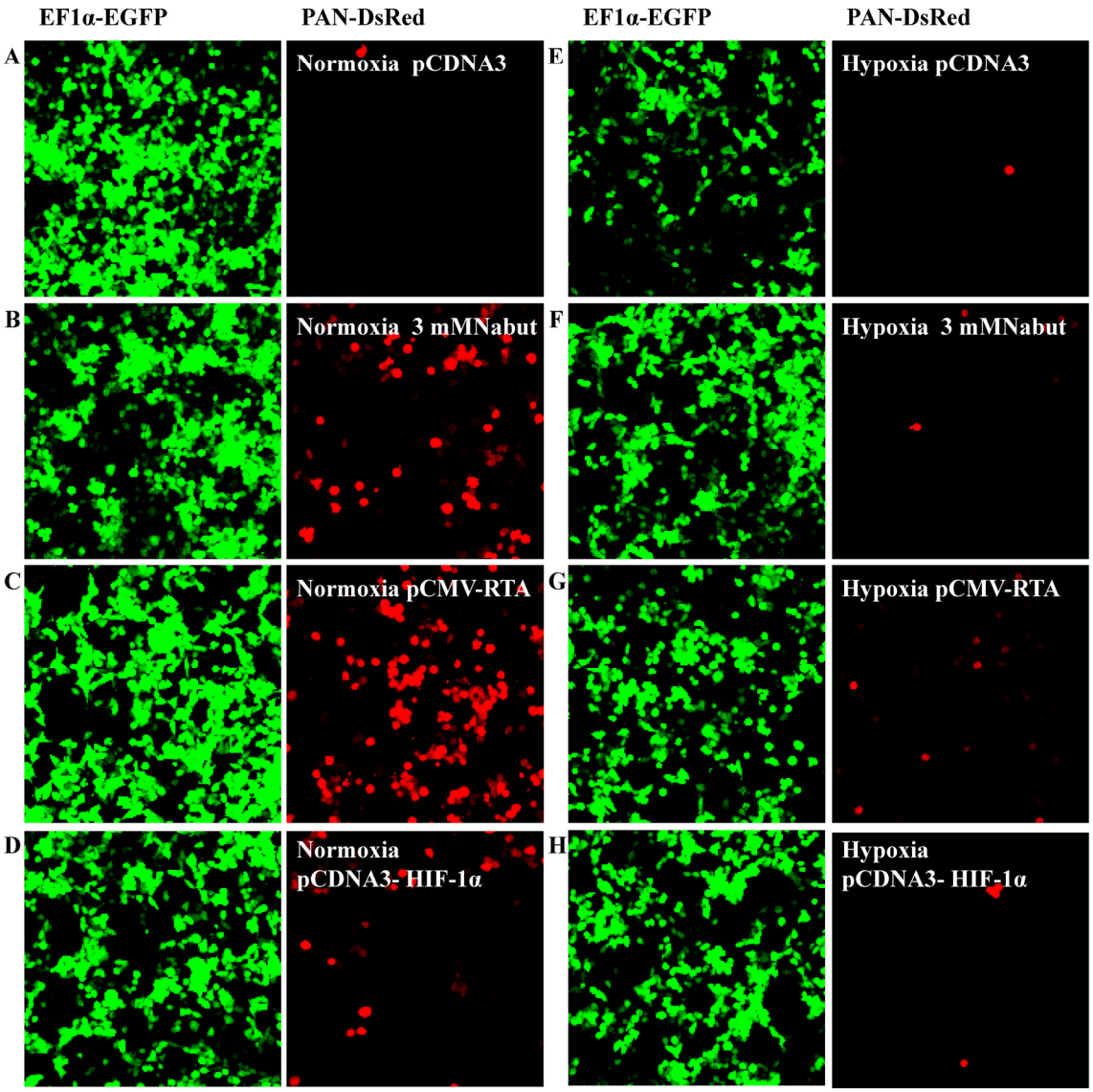


Figure 4-2 Hypoxia suppresses DsRed expression in Clone 9 cells
HEK 293T cells harbouring recombinant KSHV, rKSHV.219 (rKSHV.219 HEK 293Ts – Clone 9) were transfected with 1 µg empty vector (pCDNA3) (A and E) or stimulated to induce the KSHV lytic cycle by 3 mM sodium butyrate (NaBut) treatment (B and F) or transfected with 1 µg pCMV-RTA (C and G) or 1 µg pCDNA3- HIF-1α (D and H) and grown under normoxic (21 %O_2) or hypoxic (<3 % O_2) culture conditions. After 48 hours cells were analysed using confocal microscopy. Typical fields singly excited with 488-nm light to detect cells expressing GFP from the EF1α promoter (green) or 568 nm light to detect cells expressing DsRed from the KSHV lytic cycle PAN promoter (red) are shown magnified at x60. Clone 9 cells unstimulated or stimulated to reactivate express lower levels of DsRed under hypoxia compared to normoxic controls.

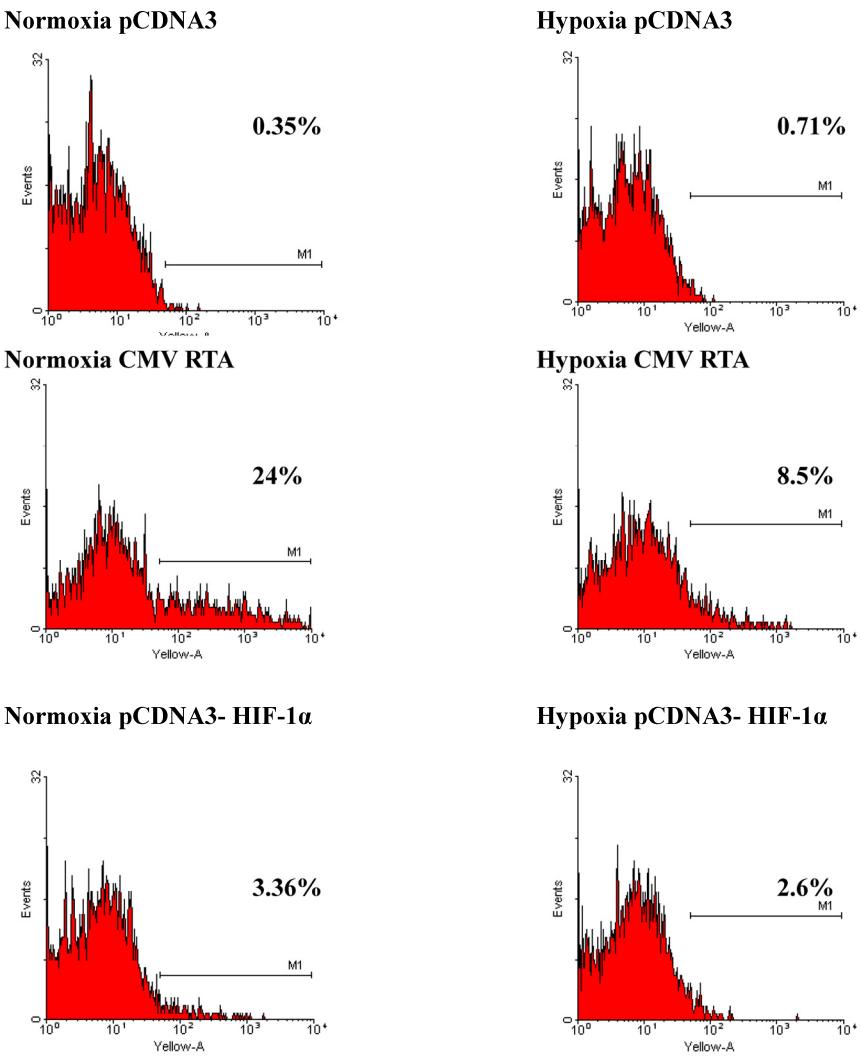


Figure 4-3 Flow cytometry analysis of Clone 9 cells after hypoxia treatment
DsRed expression of the same samples was quantified by flow cytometry. Flow cytometry results reflect the pattern of reactivation but underestimates the number of DsRed cells detected by confocal microscopy.

Previously, we have shown that XBP-1s induces the lytic cycle and DsRed expression from the PAN lytic promoter; see section 3.2.2.1. When XBP-1s was supplied to Clone 9 cells under normoxic conditions at 48 hours around 5 % of the cells, measured by FACsArray, are DsRed-positive (black bars Figure 4-4). After 48 hours under hypoxic conditions only 1 % of the Clone 9 cells expressing XBP-1s are DsRed-positive; a level similar to background levels of spontaneous PAN lytic promoter activity (grey bars Figure 4-4). After 24 hours in hypoxia and then a recovery period of 24 hours in normoxia (reoxygenation), 9 % of Clone 9 cells transfected with XBP-1s expressed DsRed (white bars Figure 4-4). Therefore, under conditions of 48 hours hypoxia Clone 9 cells are not induced to express DsRed from the PAN lytic promoter and the response to NaBut, RTA, HIF-1α and XBP-1s is reduced compared to parallel normoxic

cultures. Subjecting Clone 9 cells to hypoxic conditions followed by normoxia (reoxygenation), however, leads to DsRed expression and enhanced PAN lytic promoter activity induced by XBP-1s.

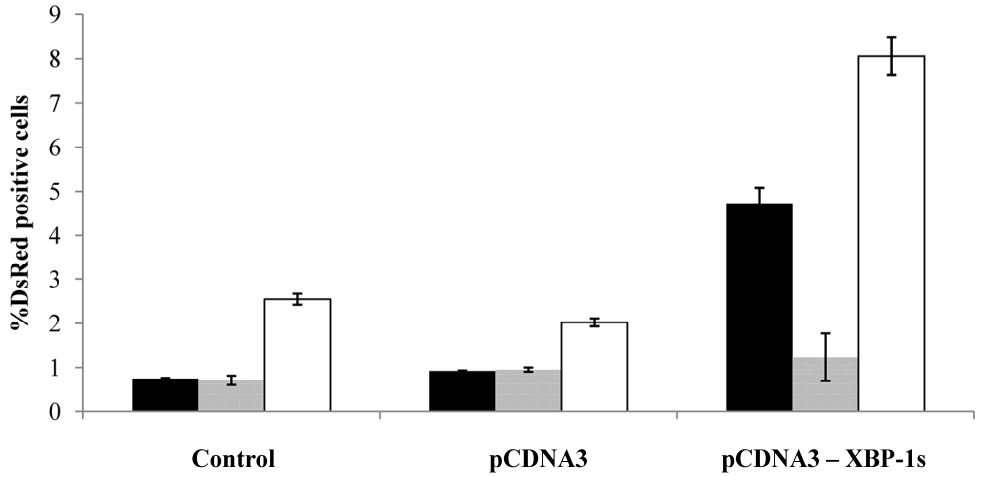


Figure 4-4 Graphical representation of FACs analysis of Clone 9 cells exposed to hypoxia
Clone 9 cells untransfected or transfected with 1 µg empty pCDNA3 or pCDNA3-XBP-1s were exposed to 48 hours of normoxia (black bars), hypoxia (grey bars) or 24 hours hypoxia followed by 24 hours normoxia (reoxygenation – white bars). Activity of the PAN lytic promoter in these cultures was measured by flow cytometry and displayed as the percentage of DsRed-positive cells in the culture. Reoxygenation of samples reveals the affect of hypoxia-induced reactivation and allows fluorescence of DsRed from the PAN lytic promoter. Columns represent mean of three independent experiments and error bars represent the standard error of the mean.

4.2.2.2 Exposure to 72 hours hypoxia or reoxygenation induces rKSHV.219 reactivation in Clone 9 cells

48 hours exposure to continued hypoxia was unable to induce DsRed expression from the PAN promoter in Clone 9 cells. However, increasing the length of hypoxic exposure to 72 hours led to increased DsRed expression compared to parallel cultures maintained at 21 % O_2 (normoxia) (Figure 4-5 A and B). The number of DsRed-expressing Clone 9 cells further increased when cells were cultured for 48 hours under hypoxic conditions then reoxygenated and grown under normoxic conditions for a further 24 hours before analysis (Figure 4-5 C). Western blot analysis was used to determine the presence of RTA in DsRed-expressing Clone 9. Under normoxic conditions RTA was not detected at 24 and 72 hours (Figure 4-6 A). Hypoxia readily induces RTA expression at 72 hours but not at 24 hours in Clone 9 cells (Figure 4-6 A). After 48 hours hypoxia followed by 24 hours normoxia (reoxygenation) RTA levels were reduced compared to 72 hours continuous hypoxia but higher than in normoxic controls (Figure 4-6 A).

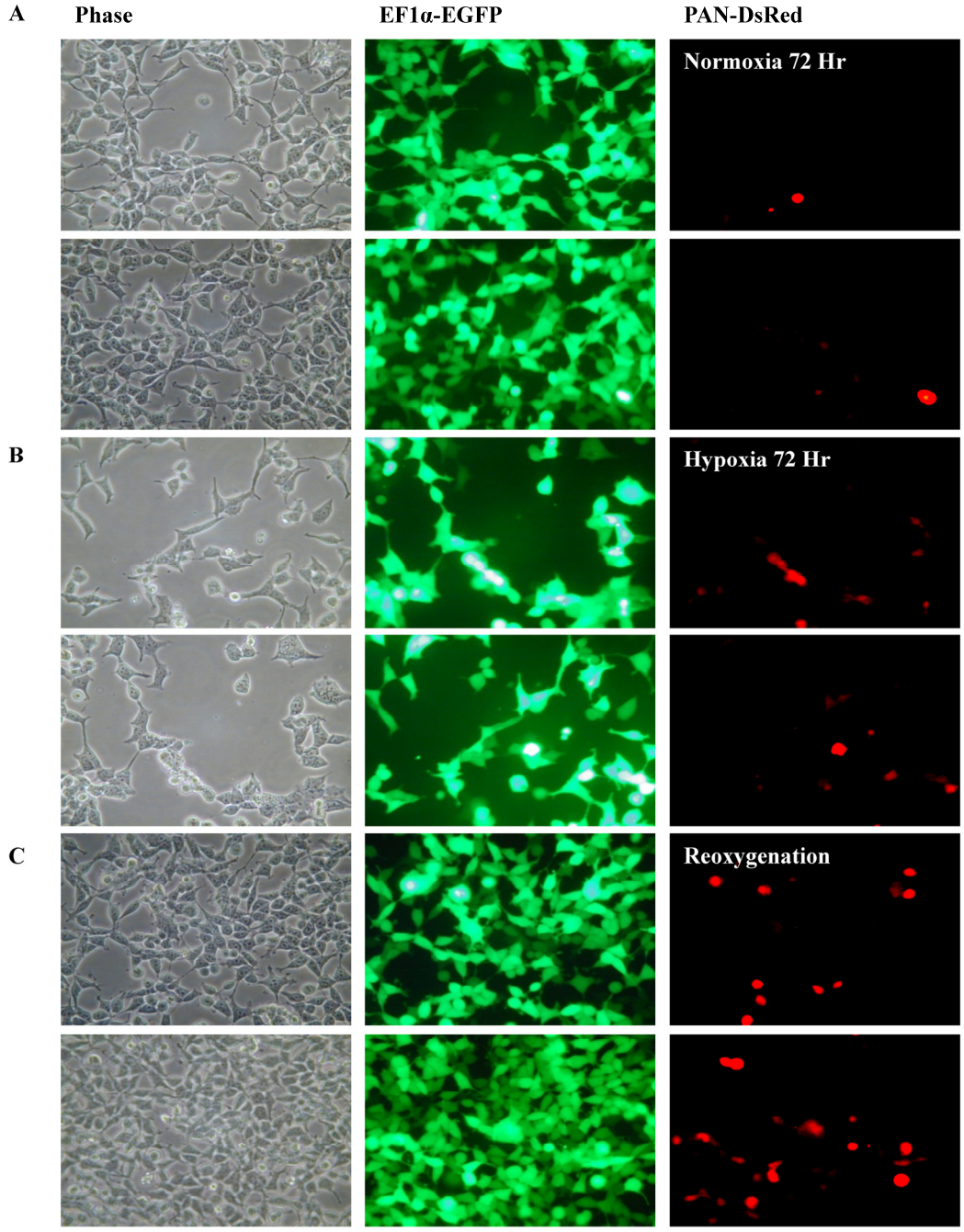


Figure 4-5 Clone 9 cells express DsRed in response to 72 hours hypoxia or reoxygenation
Photomicrographs of rKSHV.219 HEK 293T cells (Clone 9) grown under (A) normoxic (21 %O_2) or (B) hypoxic (<3 % O_2) culture conditions for 72 hours or (C) for 48 hours under hypoxia and then returned to normoxic conditions for 24 hours (reoxygenation). Two typical fields of phase, EF1α-GFP and PAN-DsRed fluorescence of each treatment are shown magnified at x20. Hypoxic and reoxygenated cultures show DsRed expression greater than parallel normoxic cultures at 72 hours.

Enhanced DsRed expression was seen in Clone 9 cells transfected with XBP-1s and subjected to reoxygenation (Figure 4-4). Due to the role of XBP-1 and KSHV reactivation previously established (see section 3.2.3) we assayed XBP-1 transcript splice status in these cells by RT PCR. Minimal XBP-1 spliced transcript is present in Clone 9 cells after 24 or 72 hours culture under normoxic

conditions (Figure 4-6 B). After 72 hours hypoxic treatment of Clone 9 cells, a strong band of spliced XBP-1 can be seen relative to 24 hours hypoxia and normoxic controls (Figure 4-6 B). When Clone 9 cells were subjected to a 24 hour period of normoxia after 48 hours hypoxia (reoxygenation) normal control levels of minimal XBP-1s are detected by RT PCR (Figure 4-6 B). Therefore, hypoxia can induce both RTA and XBP-1s but only after prolonged (72 hours) hypoxia treatment.

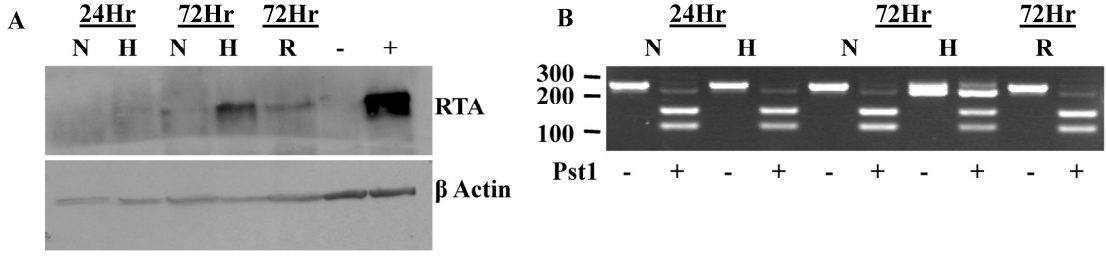


Figure 4-6 rKSHV.219 reactivation and XBP-1 splice status in Clone 9 cells in response to hypoxia or reoxygenation
(A) Western blot analysis with polyclonal rabbit anti-RTA of whole cell lysates of Clone 9 cells cultured in normoxia (N; 21 %O_2), hypoxia (H; <3 % O_2) for 24 or 72 hours or reoxygenated for 24 hours after 48 hours hypoxic exposure (R). RTA is induced by hypoxia at 72 hours and is present at a lower level in reoxygenated samples compared to cells under normoxia. β-actin acts as a loading control. (B) RT PCR amplification across the XBP-1 intron produces a 249 base pair amplicon from XBP-1u mRNA and a 223 base amplicon from XBP-s mRNA. Pst I digests only the XBP-1u amplicon resulting in two bands, whereas XBP-1s results in a single band (Wilson et al., 2007). RT PCR amplification from the total mRNA of Clone 9 cells cultured under normoxia (N; 21 %O_2), hypoxia (H; <3 % O_2) for 24 or 72 hours or reoxygenated for 24 hours after 48 hours hypoxic exposure (R) shows that XBP-1s is produced after 72 hours of hypoxia but is absent in reoxygenated samples and in cells under normoxia. In the 72 hours hypoxia sample a slower-migrating non-Pst I digestible PCR hybrid between the XBP-1s and XBP-1u products is visible similar to that previously described (Wilson et al., 2007). RT PCR for β-actin on the same RNA was used to normalise cDNA input (data not shown).

4.2.3 The rKSHV.219 JSC-1 cell model for hypoxia-induced reactivation

4.2.3.1 Exposure to 72 hours hypoxia or reoxygenation induces rKSHV.219 reactivation in rKSHV.219 JSC-1 cells

A more relevant model for KSHV reactivation in response to hypoxic treatment is to use the PEL cell line rKSHV.219 JSC-1 (a kind gift from the lab of Jeff Vieira); which contains an identical rKSHV.219 to the HEK 293T cells used in the previous sections. rKSHV.219 JSC-1 cells were exposed to the established hypoxic conditions. After 72 hours hypoxic treatment DsRed expression from the PAN lytic promoter was marginally increased above normoxic controls (Figure 4-7 B). As seen with the Clone 9 cells, a recovery period of 24 hours in normoxic conditions after 48 hours of hypoxia led to an increase in DsRed-

positive cells as seen by microscopy. This was quantified by FACsArray analysis to around 8 % of the cells (Figure 4-7 A and B). Therefore the rKSHV.219 JSC-1 model for reactivation responds to the hypoxic culture conditions in the same way as Clone 9 cells, although possibly to a lesser extent.

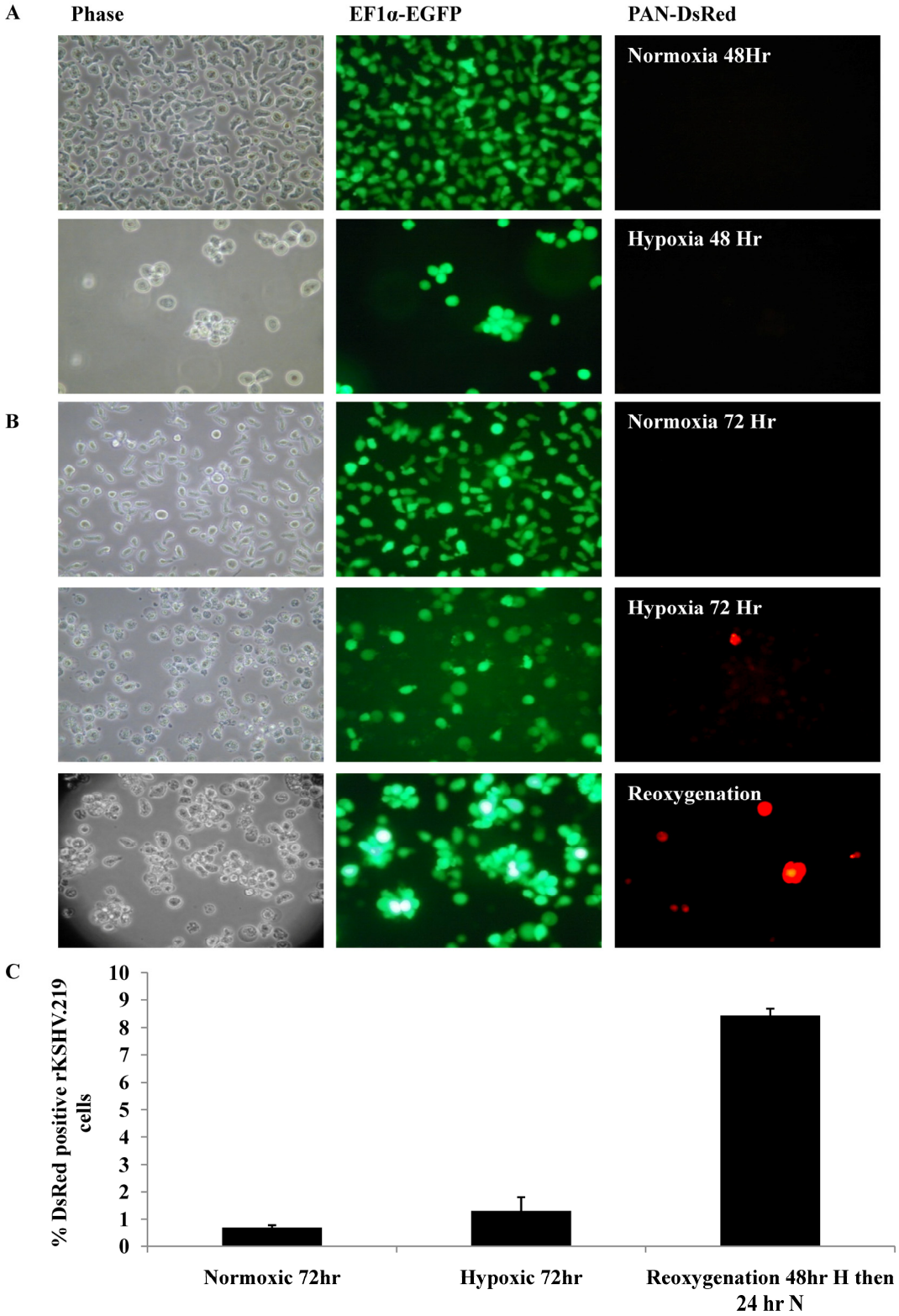


Figure 4-7 rKSHV.219 JSC-1 cells express DsRed in response to 72 hours hypoxia or reoxygenation

Photomicrographs of the PEL cell line rKSHV.219 JSC-1 grown under normoxic (21 %O_2) or hypoxic (<3 % O_2) culture conditions for (A) 48 or (B) 72 hours or for 48 hours under hypoxia and then returned to normoxic conditions for 24 hours (reoxygenation). Typical fields of phase, EF1α-GFP and PAN-DsRed fluorescence of each treatment are shown magnified at x20. 72 hr hypoxic treated and reoxygenated cultures show DsRed expression greater than 48 hr hypoxic cultures and parallel normoxic cultures at 72 hours. (C) The activity of the PAN lytic promoter in the 72 hour cultures above was measured by flow cytometry and displayed as the percentage of DsRed-positive cells in the culture. Reoxygenation of samples reveals the affect of hypoxia-induced reactivation and allows fluorescence of DsRed from the PAN lytic promoter. Columns represent mean of nine independent experiments and error bars represent the standard error of the mean.

4.2.4 Determining the role of HIF-1 and XBP-1 in hypoxia-induced reactivation

4.2.4.1 Hypoxia stabilises HIF-1α and activates XBP-1 in PEL

Hypoxia is able to induce both HIF-1α stabilisation and XBP-1 splicing, and both transcription factors can transactivate the ORF50 promoter through 'ACGT' core containing XREs and HREs respectively (see section 3.2.4.1). Therefore, we examined the ability of hypoxia to induce both transcription factors in PEL. HIF-1α is stabilised and activated at oxygen concentrations below 3 % in a variety of cell lines (Koumenis and Wouters, 2006). Under conditions of acute hypoxia (<3 % O_2) HIF-1α is stabilised and readily detected in the PEL cell line rKSHV.219 JSC-1 (Figure 4-8 A). Levels of HIF-1α are increased by 48 hours of hypoxia compared to cells cultured in normoxia and are robustly expressed after 72 hours of hypoxic treatment (Figure 4-8 A). We note, however, that in our hands PEL do not consistently express detectable HIF-1α under normoxia in contrast to work carried out by Cai *et. al* (Cai et al., 2007). We have previously shown that XBP-1 is present in PEL as the unspliced, inactive transcript which can be activated to the spliced bZIP-containing transcript by the reducing agent DTT (Wilson et al., 2007). We have seen an increase in XBP-1 spliced transcript after 72 hours hypoxia treatment in rKSHV.219 HEK 293T cells, Clone 9 (see section 4.2.2.2). XBP-1 has also been shown to be activated by hypoxia in cardiac myocytes (Thuerauf et al., 2006). Consistent with these data, exposure of rKSHV.219 JSC-1 cells to hypoxia leads to significant conversion of XBP-1 to the spliced, active form (XBP-1s) (Figure 4-8 B). Therefore hypoxia induces both HIF-1α and XBP-1s transcription factors in PEL.

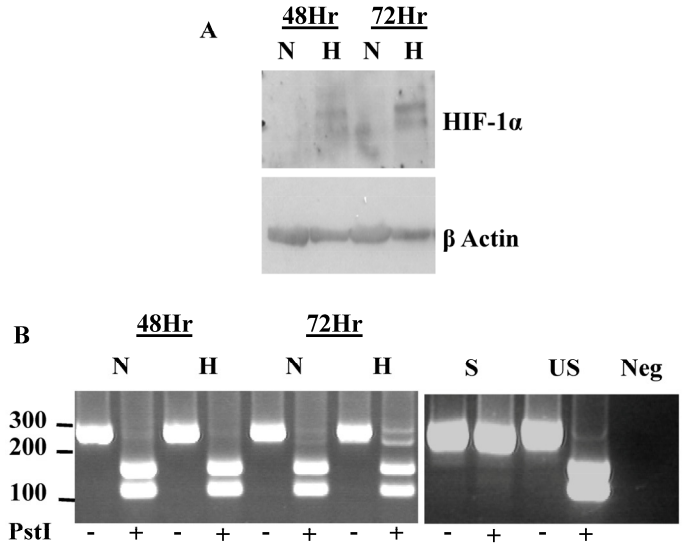


Figure 4-8 Hypoxia-induced HIF-1α stabilisation and XBP-1 splicing in PEL
(A) Western blot analysis of whole cell lysates of the PEL cell line rKSHV.219 JSC-1 cultured in normoxia (N; 21 %O_2) or exposed to 48 or 72 hours of hypoxia (H; <3 % O_2) with anti-HIF-1α antibody (BD Transduction Labs). HIF-1α is stabilised in rKSHV.219 JSC-1 cells in response to hypoxia at 48 and 72 hours compared to normoxia, β-actin acts as loading control. (B) RT PCR amplification from the total mRNA of the rKSHV.219 JSC-1 cell line cultured under normoxia (N; 21 %O_2) or exposed to hypoxia (H; <3 % O_2) for 48 or 72 hours shows that XBP-1s is produced after 72 hours of hypoxia but is absent in cells under normoxia. In the 72 hours hypoxia sample a slower-migrating non-Pst I digestible PCR hybrid between the XBP-1s and XBP-1u products is visible similar to that previously described (Wilson et al., 2007). RT PCR for β-actin on the same RNA was used to normalise cDNA input (data not shown).

4.2.4.2 Hypoxia induces the KSHV lytic cycle in PEL

The effect of hypoxia and hypoxia mimics, such as cobalt chloride, on KSHV lytic cycle induction has been demonstrated (Cai et al., 2006a; Davis et al., 2001; Haque et al., 2003). We have also shown that low oxygen conditions induce XBP-1s and RTA expression in rKSHV.219 HEK 293Ts, Clone 9 (4.2.2.2). We therefore investigated whether KSHV reactivation is seen in PEL under the same low oxygen conditions. Under normoxia RTA is not significantly expressed rKSHV.219 JSC-1 (Figure 4-9 A), wild-type JSC-1 or HBL-6 cells (Figure 4-9 D). RTA expression is readily induced in all PEL cell lines tested in response to hypoxia treatment (Figure 4-9 A and D). RT PCR for the spliced KSHV lytic cycle transcript ORF29a/b shows that KSHV enters the full lytic cycle (Figure 4-9 B) and ORF37 qPCR for extracellular KSHV viral genome confirms production of virus in response to hypoxia in both PEL cell lines JSC-1 and HBL-6 (Figure 4-9 C). Therefore RTA induced by low oxygen conditions is able to induce the full lytic cycle of KSHV in PEL.

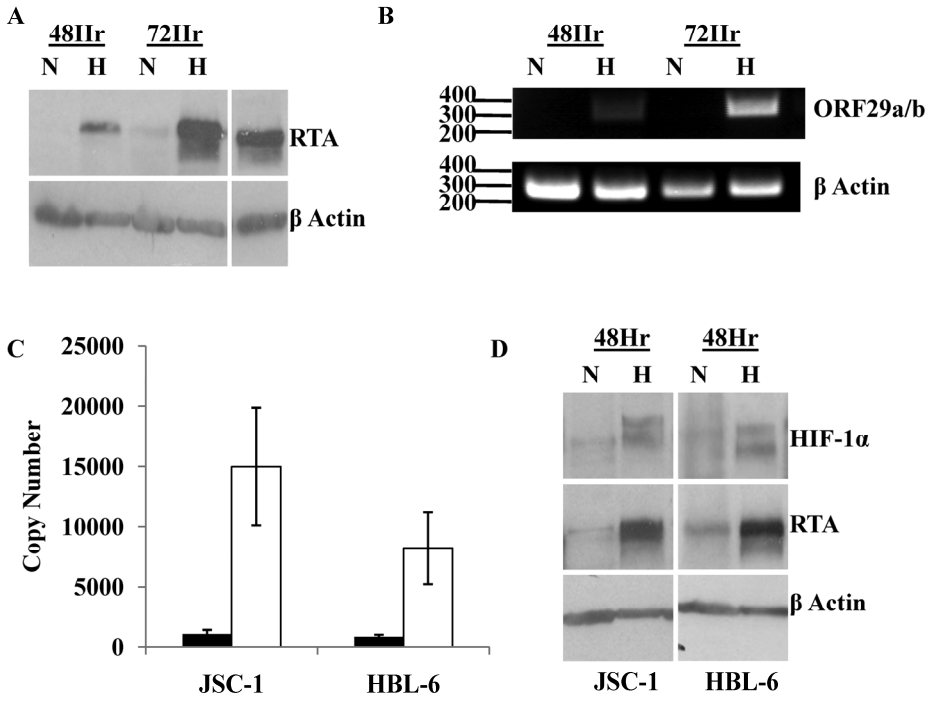


Figure 4-9 Hypoxia leads to the induction of the full KSHV lytic cycle in PEL
(A) Western blot analysis of whole cell lysates of the PEL cell line rKSHV.219 JSC-1 cultured in normoxia (N; 21 %O_2) or exposed to 48 or 72 hours of hypoxia (H; <3 % O_2) with polyclonal rabbit anti-RTA. RTA is induced by hypoxia at 48 hours and further increased at 72 hours exposure compared to cells under normoxia. β-actin acts as a loading control. (B) Total cellular RNA was isolated from rKSHV.219 JSC-1 cultured under normoxic or hypoxic conditions for 48 and 72hours. RT PCR and gene specific primers for the spliced lytic transcript, ORF29a/b, were used to amplify a 300bp product from cDNA. PCR product is seen only in samples exposed to hypoxia (H; <3 % O_2). RT PCR for β-actin on the same RNA was used to normalise cDNA input. (C) qPCR for ORF37 from KSHV virion DNA extracted from the supernatants of JSC-1 and HBL-6 cells cultured for 72 hours in normoxia (N; 21 %O_2, black bars) or Hypoxia (H; <3 % O_2, white bars). Copy number is determined using an ORF37 standard curve and normalised for total DNA input. Hypoxia leads to a significant increase in virus copies present in the supernatant. (D) Western blots for HIF-1α and RTA of whole cell lysates of the corresponding cells to the supernatants used in C. At 48 hours - shows samples are HIF-1α-positive and RTA is induced.

4.2.4.3 The RTA promoter has the potential to respond to HIF-1α and XBP-1s

The RTA promoter can be induced by the transcription factors XBP-1s and HIF-1α (Cai et al., 2006a) (Figure 4-10 A). The response elements of these transcription factors also contain essentially the same core sequence 'ACGT'. We therefore determined if 'ACGT' elements present in the OR50 promoter could respond to both transcription factors. A series of truncated constructs of the RTA promoter fused to a luciferase reporter were used to isolate HRE2 from XRE (HRE4) (Figure 4-10 B). Expression of RTA efficiently induced luciferase activity from the full-length and truncated ORF50 promoters (Figure 4-10 C). HIF-1α was also able to drive luciferase expression from the full-length

promoter as well as the HRE-containing truncated promoters as previously seen (Cai et al., 2006a). However, XBP-1s is able to activate expression of luciferase from the full-length ORF50 reporter and the XRE- (HRE-4)-containing promoter only. Therefore, the RTA promoter of KSHV can be transactivated by both HIF-1α and XBP-1s and both factors act through ACGT binding sites.

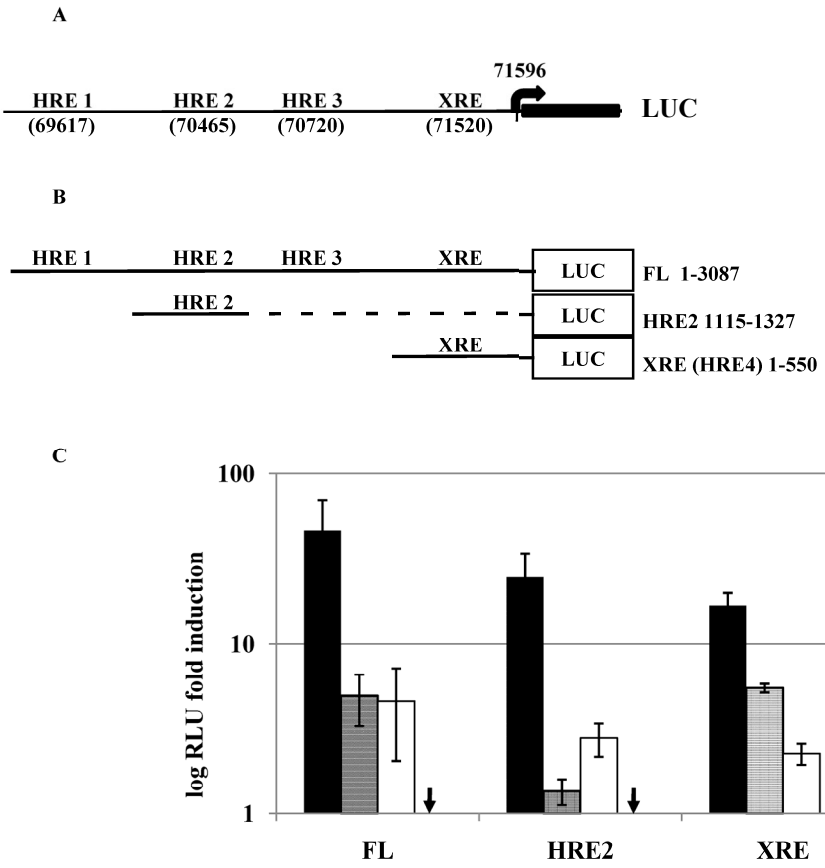


Figure 4-10 The KSHV RTA promoter responds to both XBP-1 and HIF-1α
(A) A schematic of the predicted hypoxia-responsive elements (HREs) in the KSHV RTA promoter region (adapted from (Cai et al., 2006a)). (B) Schematic of reporter plasmids pRpluc1-3087 containing a 3-kb sequence upstream of the RTA transcriptional start that drives the expression of firefly luciferase (Cai et al., 2006a) as well as truncated promoters named pRpluc 1115-1327 (HRE2 only) and pRpluc1-550 XRE (HRE4) only. (C) A fixed amount of the reporter plasmids were transfected into HEK 293T cells together with pIG (empty vector control - ↓), RTA-expressing (pCMVRTA – black bars), XBP-1s-expressing (pXBPsIG – striped bars) or HIF-1α-expressing (pHIF-1αIG – white bars) plasmids normalised by plasmid copy number. Promoter activity levels are expressed as the log of relative light units (logRLU) relative to the reporter plus pIG control plasmid. Columns represent the mean of three independent experiments and error bars represent standard error of the mean. HIF-1α acts on all reporters containing HRE2 and XRE (no significant difference 95% CFI) while XBP-1s acts on the full-length and XRE only promoter but not HRE2 only. Activity of XBP-1s on the HRE2 only promoter found to be significantly different to it's activity on the XRE only promoter $p<0.001$.

4.2.4.4 XBP-1s contributes to the RTA induction by hypoxia

As both HIF-1α and XBP-1s are activated by hypoxia at similar times to RTA induction, we wished to determine if one transcription factor was dominant for inducing KSHV lytic reactivation under hypoxia. We used the short hairpin

interfering RNA (shRNA) to XBP-1 previously described in section 3.2.3.1 and produced shRNAs to HIF-1α. These hairpins led to a reduction in HIF-1α protein levels when HIF-1α protein was overexpressed in HEK 293Ts cells (Figure 4-11). The shRNA targeted to the nucleotide region 1545 of HIF-1α mRNA sequence (NM_001530) acted more efficiently to reduce HIF-1α protein levels than shRNA targeting the nucleotide region around 1589, and therefore the former was used in further experiments and named shHIF-1α.

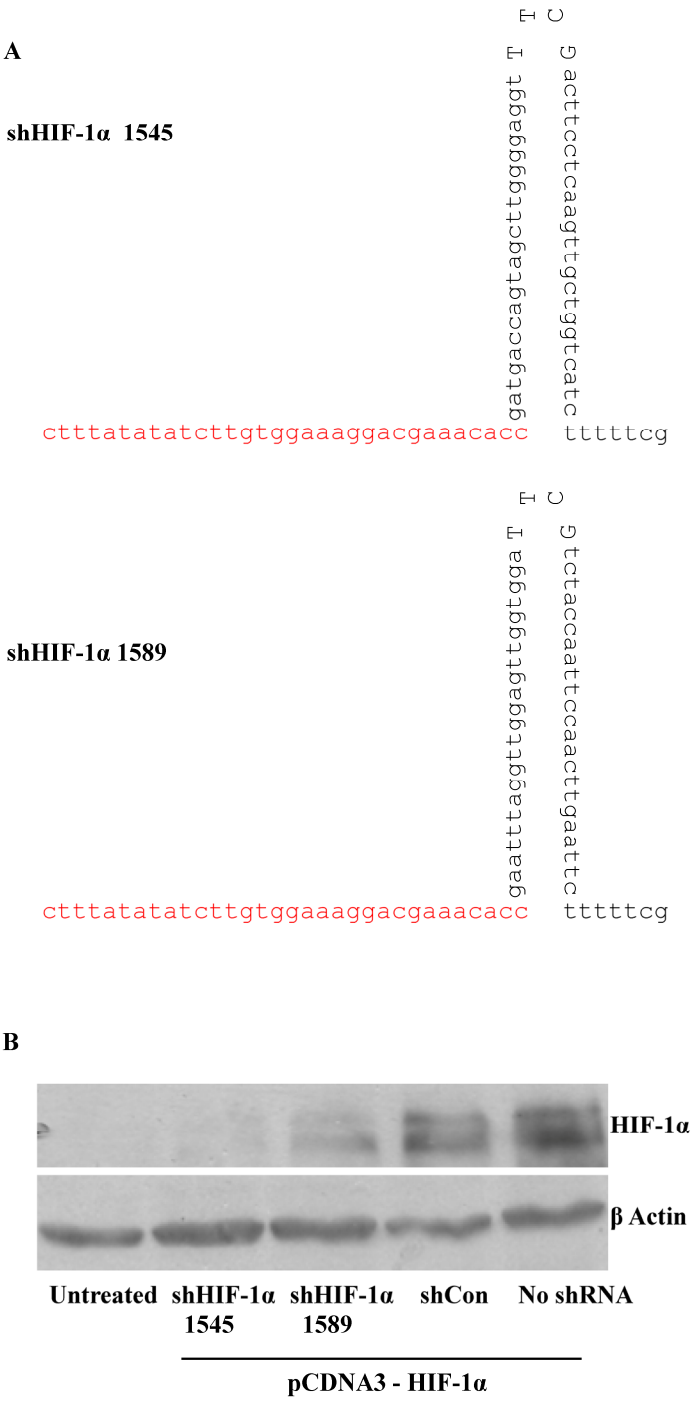


Figure 4-11 Production of shRNA to HIF-1α

(A) Sequences and probable structures of shRNAs targeting two different regions of HIF-1α mRNA. Numbering corresponds to sequence (NM_001530). (B) shRNA ability to knock down target protein levels was demonstrated by co-transfection of HEK 293T cells with 1μg of target expressing plasmid (pCDNA3-HIF-1α) and 3 μg of relevant/irrelevant control hairpins in a pGEMT backbone. Western blot for target protein using anti-HIF-1α (BD transduction labs) reveal both shRNA targeting HIF-1α are able to reduce target protein level substantially compared to the irrelevant control shRNA. β-actin acts as a loading control. shRNA 1545 HIF-1α was used for further experiments.

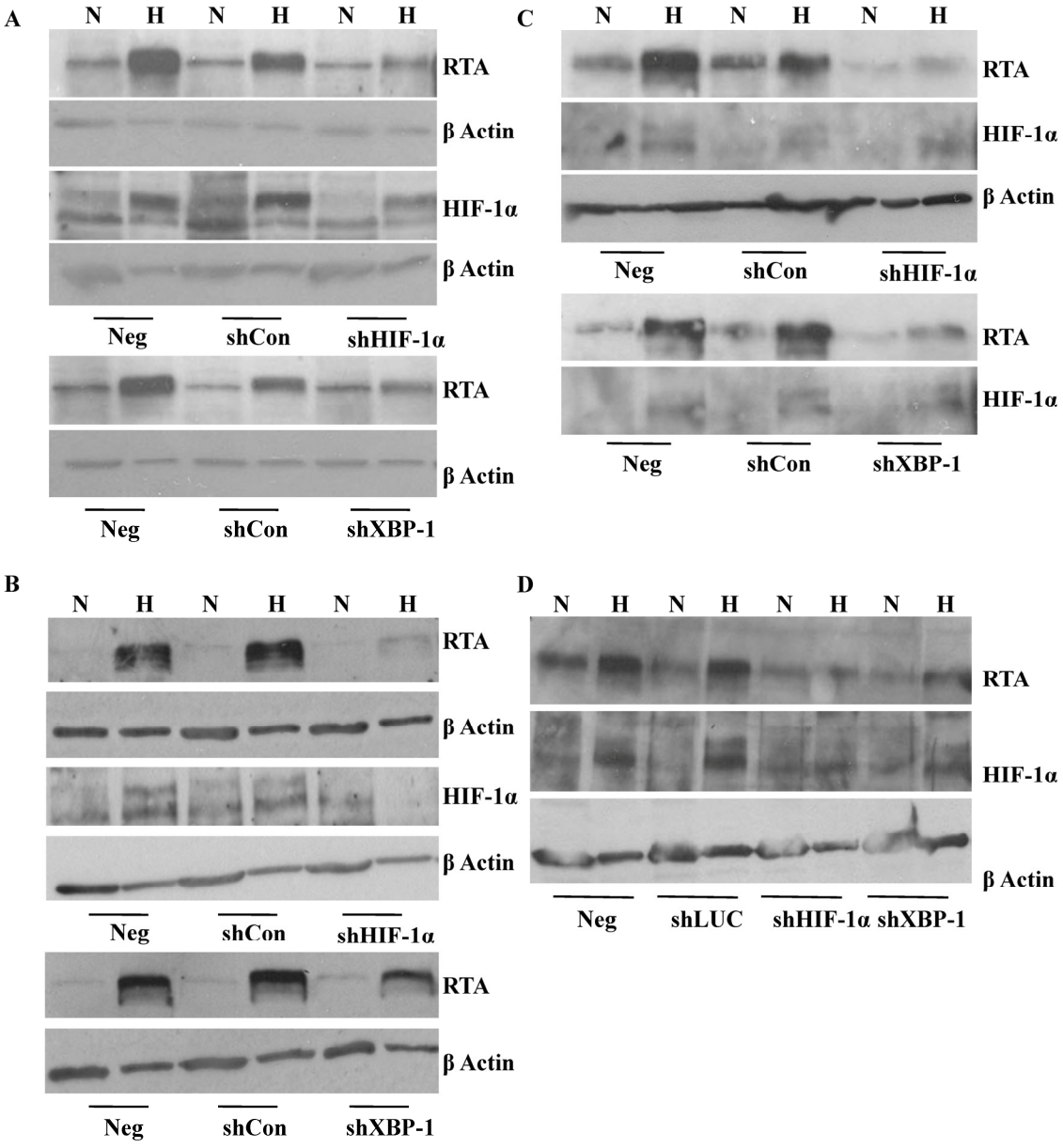


Figure 4-12 XBP-1s involvement in RTA induction by hypoxia
(A) Clone 9 (B) rKSHV.219 JSC-1, (C) JSC-1 and (D) HBL-6 cells stably transduced with lentiviruses containing shRNA to XBP-1, HIF-1α and irrelevant control independently were cultured in normoxia (N-21 %O_2) or hypoxia (H- <3 % O_2) for 72 hours. Western blot analysis of whole cell lysates was performed for HIF-1α indicating its upregulation in hypoxic samples and attenuated expression in those cells expressing the shRNA to HIF-1α. The same samples probed with anti-RTA show the induction of RTA in response to hypoxia which is reduced in the presence of shRNA to HIF-1α or XBP-1s. β-actin acts as a loading control.

The cell line rKSHV.219 HEK 293T, Clone 9 (Figure 4-12 A) or the PEL cell lines rKSHV.219 JSC-1 (Figure 4-12 B), JSC-1 (Figure 4-12 C) and HBL-6 (Figure 4-12 D) were transduced with lentivirus expressing hairpins targeting XBP-1, HIF-1α or a control with an input equivalent to a MOI 5 on HEK 293-T cells. Stable cell lines were then produced and maintained using puromycin selection. Cells were exposed to hypoxia or normoxia for 72 hours before being sampled. Western blot analysis shows that decreasing the levels of HIF-1α under hypoxia clearly results in attenuated RTA induction (Figure 4-12). The shRNA to XBP-1s also led to a reduction in RTA expression under the same hypoxic conditions (Figure 4-12). The presence of shRNA does not interfere with the processing of XBP-1 mRNA (data not shown). These data suggest that both HIF-1α and XBP-1s contribute to the lytic cycle induction of KSHV under hypoxia.

4.2.4.5 HIF-1α overexpression under normoxia does not lead to RTA induction in PEL

Due to the stabilisation of HIF-1α in PEL and KS lesions (Cai et al., 2006b; Carroll et al., 2006), we wanted to determine the contribution of HIF-1α to KSHV lytic cycle induction under normoxia. We transduced the PEL cell lines JSC-1, HBL-6 and BC-3 with lentivirus expressing HIF-1α, XBP-1s or empty vector at an input equivalent to a MOI 5 on HEK 293-T cells.

Overexpression of HIF-1α from the lentivirus vector led to HIF-1α protein expression in all PEL cell lines (Figure 4-13 A). Previously, HIF-1α overexpression resulted in transactivation of the ORF50 promoter in HEK 293T cells and DsRed expression from the PAN lytic promoter in rKSHV.219 HEK 293T cells (Figure 4-13 B and section 4.2.2.1). However, RTA was only induced in PEL cells overexpressing XBP-1s under normoxia (Figure 4-13 B). The overexpression of HIF-1α in PEL did not reactivate KSHV under normoxia. Therefore, only XBP-1s is able to reactivate KSHV in PEL cells under conditions of normoxia and hypoxia.

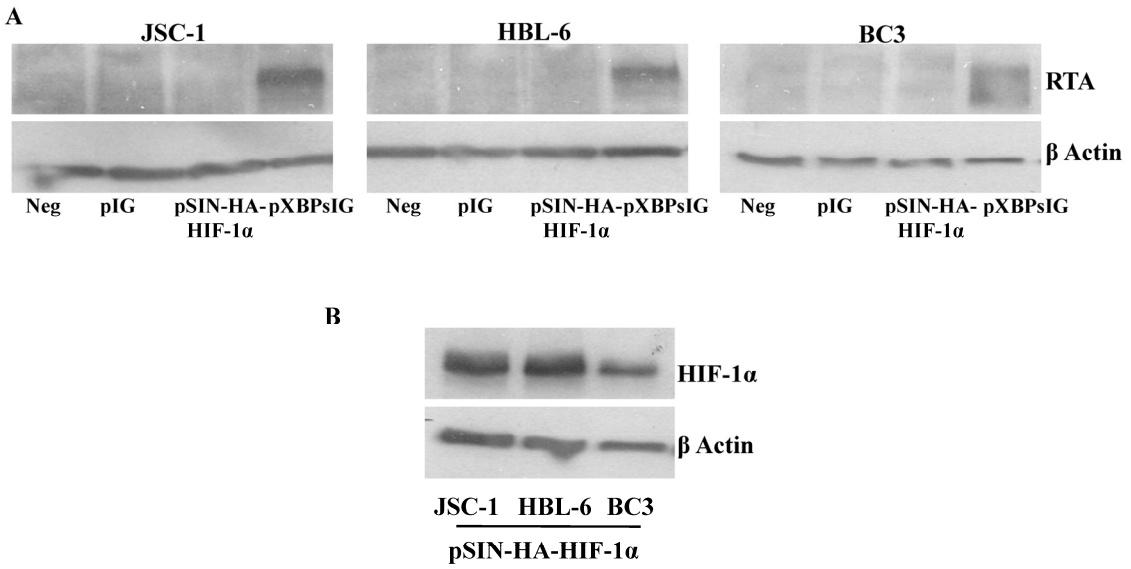


Figure 4-13 Overexpression of HIF-1α in PEL under normoxia
(A) Whole cell lysate RTA western blot of JSC-1, HBL-6 and BC3 cells transduced with empty vector (pIG) or HIF-1α-expressing (pSIN-HA-HIF-1α)) or XBP-1s-expressing (pXBPsIG) at an input equivalent to a MOI 5 on HEK 293-T cells. RTA is induced only in the presence of XBP-1s expression (pXBPsIG) under normoxia. (B) Anti-HIF-1α western blot of whole cell lysates of JSC-1, HBL-6 and BC3 cells transduced with lentivirus expressing HIF-1α. β-actin acts as a loading control.

4.2.4.6 Co-expression of HIF-1α and XBP-1s under normoxia in PEL

To determine whether normoxic expression of HIF-1α required the presence of XBP-1s in order to induce RTA expression in PEL, we overexpressed both proteins simultaneously in the PEL cell line JSC-1.

As seen before, HIF-1α overexpression in JSC-1 did not induce RTA expression under normoxia (Figure 4-13). Expression of HIF-1α in conjunction with XBP-1s did not increase the level of RTA protein over and above the level induced in the presence of XBP-1s alone (Figure 4-14 A). Expression of both XBP-1s and HIF-1α in HEK 293Ts led to a 3.2 fold increase in the expression of luciferase from a full-length ORF50 promoter (Figure 4-14 B). XBP-1s and HIF-1α, when expressed individually, caused a 2.2 and 2.6 fold increase in RTA promoter activity respectively (Figure 4-14 B). The presence of XBP-1s therefore does not allow HIF-1α to induce KSHV lytic reactivation under normoxia.

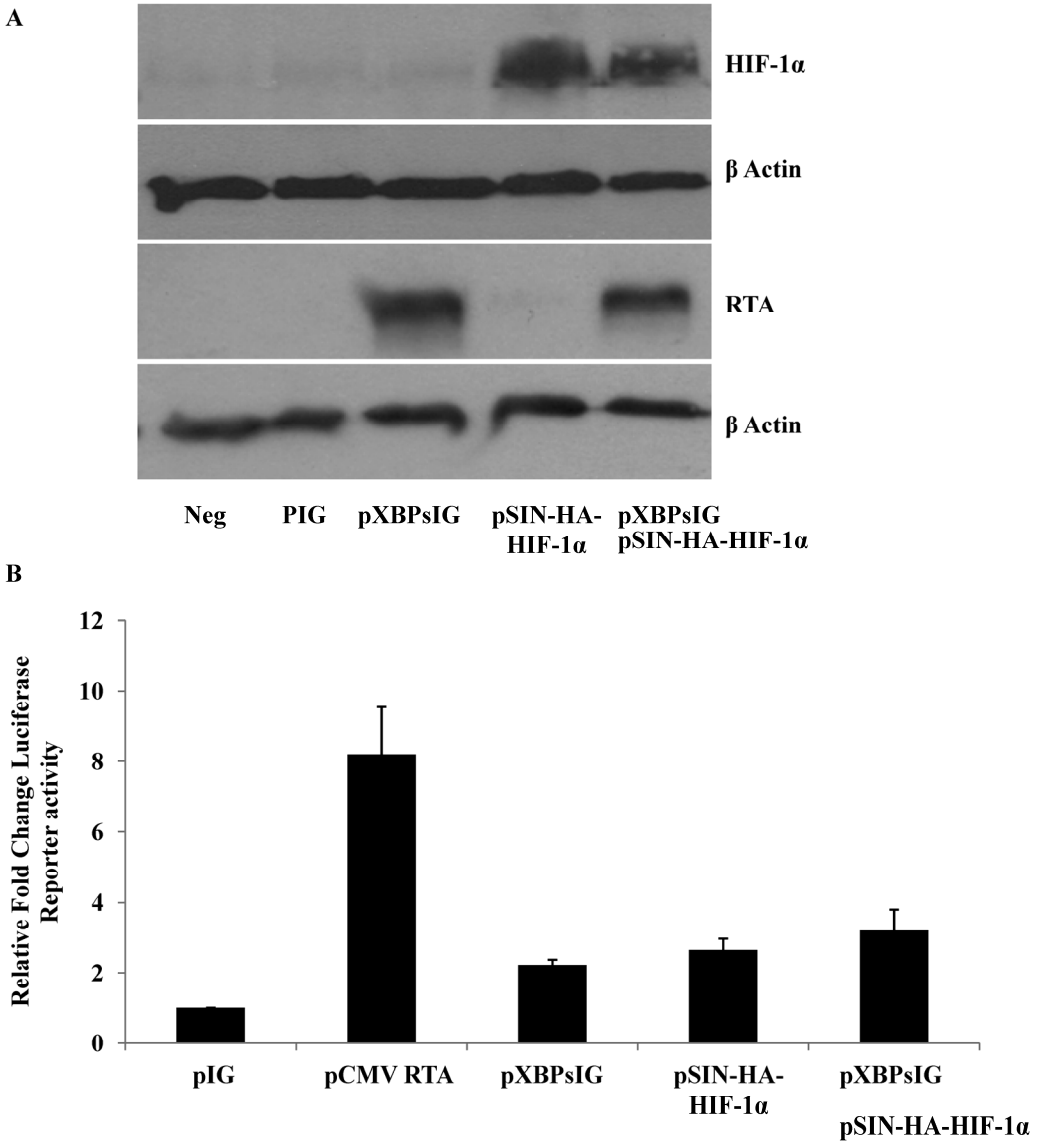


Figure 4-14 Co-expression of HIF-1α and XBP-1s under normoxia
(A) Whole cell lysate anti-HIF-1α and RTA western blot of JSC-1 cells transduced with empty vector (pIG), HIF-1α-expressing (pSIN-HA- HIF-1α), XBP-1s-expressing (pXBPsIG) at an input equivalent to a MOI 5 on HEK 293-T cells or both pSIN-HA- HIF-1α and pXBPsIG. RTA is induced only in the presence of XBP-1s expression (pXBPsIG) under normoxia and is not further induced when both XBP-1s and HIF-1α are expressed. β-actin acts as a loading control. (B) HEK 293T cells were transfected with a *Renilla*-expressing control plasmid (phRL-null) plus the ORF50 promoter *Firefly* luciferase reporter plasmid (pRpluc1-3087+s) together with pIG (control) or RTA-expressing (pCMVRTA) or XBP-1s-expressing (pXBPsIG), or HIF-1α-expressing (pSIN-HA- HIF-1α) or the last two plasmids in combination. *Firefly* luciferase activity was determined 48 hours post-transfection and normalised to *Renilla* luciferase (phRL-null) activity and plotted as a fold change relative to empty vector control pIG. Columns represent the mean of three independent experiments and error bars represent standard error of the mean. No significant difference was found when plasmids were used in combination to the 95% confidence level.

4.2.5 Hypoxia induces EBV BZLF-1 expression in both BL and PEL

It has been previously demonstrated that hypoxic culture of B95-8 cells, a marmoset B-cell line transformed with EBV, induced BZLF-1 expression and lytic replication (Jiang et al., 2006). Burkitt's Lymphoma (BL) is an EBV associated B-cell lymphoma, we therefore took BL derived cell lines and

monitored the expression of BZLF-1 in response to the same hypoxic conditions. BZLF-1 expression is dramatically up regulated in response to low oxygen conditions in both Mutu and Daudi cell lines compared to normoxic controls. No EBV reactivation is seen in Raji cells where EBV is known to be defective (Figure 4-15 A). The PEL cell line JSC-1 is co-infected with EBV, therefore we looked for BZLF-1 expression in the same samples that had displayed KSHV reactivation under hypoxic conditions (see section 4.2.4.4). BZLF-1 was strongly induced in all hypoxic samples including those stably transduced with control shRNA-GFP or shRNA to HIF-1α (Figure 4-15 A). In JSC-1 samples expressing shRNA to XBP-1s, BZLF-1 can be seen by western blot under normoxic conditions and is further accumulated after hypoxic treatment (Figure 4-15 A). The EBV present in PEL cells is also induced into the lytic cycle by hypoxia, this appears however to be independent of HIF-1α, while the role of XBP-1s remains complicated.

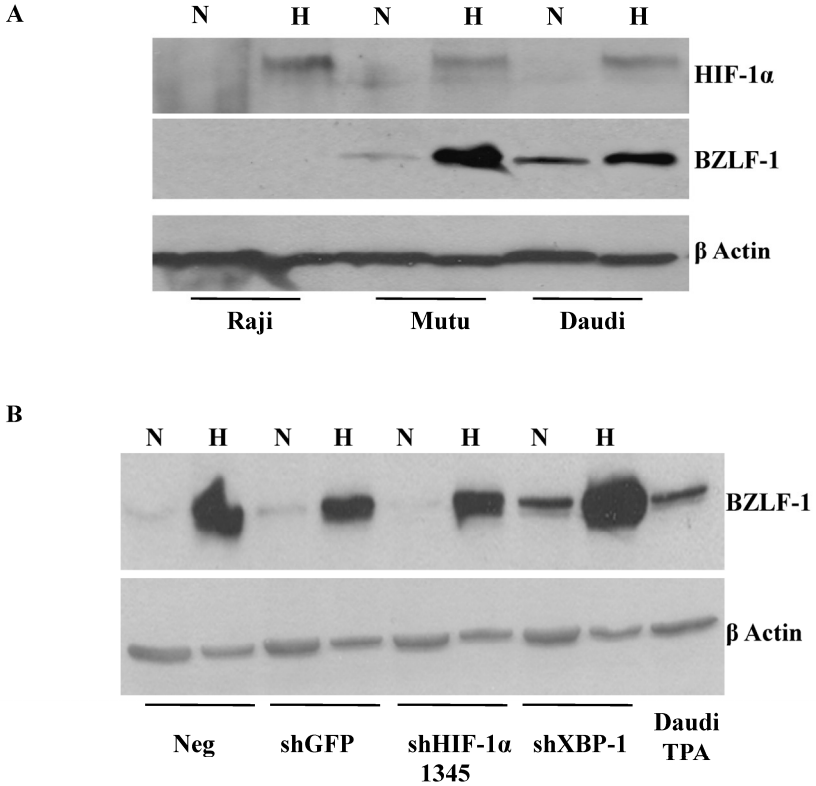


Figure 4-15 EBV reactivation in response to hypoxia
(A) Western blot analysis of whole cell lysates of the BL cell lines Raji, Mutu and Daudi were cultured in normoxia (N; 21 %O_2) or exposed to 72 hours of hypoxia (H; <3 % O_2) with anti-HIF-1α antibody (BD transduction labs). HIF-1α is stabilised in all cell lines in response to hypoxia at 72 hours compared to normoxia. BZLF-1 western blot analysis of the same samples shows BZLF-1 is robustly upregulated in all hypoxic treated samples compared to normoxic samples except in the EBV defective Raji cell line. β-actin acts as loading control. (B) JSC-1 cells stably transduced independently with lentiviruses containing shRNA to XBP-1, HIF-1α and irrelevant control, GFP, were cultured in normoxia (N-21 %O_2) or hypoxia (H- <3 % O_2) for 72 hours. Western blot analysis of whole cell lysates was performed for BZLF-1 indicating its upregulation in all hypoxic samples (except for EBV defective Raji cell samples) relative to normoxic parallel cultures. β-actin acts as a loading control.

4.3 Discussion

Here we have established a role of both transcription factors XBP-1s and HIF-1α in hypoxia-induced KSHV reactivation. Hypoxia was previously identified as an inducer of KSHV lytic replication (Davis et al., 2001), and HREs, required for the transcription factor activity of HIF-1α, are present within the promoter regions of KSHV viral genes including ORF50/RTA (Haque et al., 2003). However, studies of RTA promoter activity in response to HIF-1α over expression (Haque et al., 2003) (Jiang et al., 1997b) or hypoxia mimics show minimal or cell type specific activation by HIF-1α under otherwise normoxic culture conditions. Under low oxygen conditions however the RTA promoter is active. Hypoxia mimics such as cobalt chloride and desferoxamine are iron chelators which act to prevent HIF-1α degradation and are often used in KSHV RTA induction studies (Wang and Semenza, 1993b) (Carroll et al., 2006) (Haque et al., 2003). Whilst this may lead to the expression of HIF-1α dependent genes (Costa-Giomi et al., 1990; Wang and Semenza, 1993b) hypoxia mimics do not accurately represent conditions of low oxygen (authentic hypoxia - see section 1.5.1.1). Combining previous studies together, data suggests that either HIF-1α is not sufficient for the induction of KSHV lytic cycle or that authentic hypoxia is required, possibly causing the induction of other factors that may promote KSHV reactivation.

HIF-1α is the major transcriptional activator under hypoxic conditions and is known to regulate over 100 genes involved in the cellular response to stress induced by low oxygen levels (Semenza and Wang, 1992) (Manalo et al., 2005). However, HIF-1α independent responses to hypoxia have been reported and generally include processes that require oxygen or energy consumption, for example protein folding (Wykoff et al., 2000) (Tu et al., 2000) (Tu and Weissman, 2002). The accumulation of misfolded protein under hypoxic conditions leads to the activation of the unfolded protein response (UPR) in a HIF-1α independent manner (Koumenis et al., 2002) (Ameri et al., 2004) (Koumenis and Wouters, 2006). XBP-1 is a major component of the UPR and is able to induce KSHV reactivation (see chapter 3) (Wilson et al., 2007) (Yu et al., 2007a). Therefore, to establish the combined effects of XBP-1s and HIF-1α on KSHV reactivation under hypoxia true, low oxygen, hypoxia conditions are

essential (Feldman et al., 2005) (Romero-Ramirez et al., 2004). Fundamentally the HIF-1α-independent hypoxia-induced responses are absent in studies that employ hypoxia mimics or HIF-1α overexpression as a surrogate for hypoxic conditions. Using established techniques we were able to achieve physiologically relevant, low oxygen, hypoxic culture conditions that led to the accumulation of HIF-1α for our study (Figure 4-1).

When rKSHV.219 HEK 293T cells, Clone 9, were exposed to these hypoxic conditions for 48 hours, DsRed expression was not induced (Figure 4-2 A and E). This suggested that these hypoxic conditions do not induce KSHV lytic reactivation in contrast to previously published results (Davis et al., 2001). However, known lytic cycle inducers such as, NaBut, RTA and XBP-1s also did not induce DsRed expression when introduced under hypoxia (Figure 4-2). The fact that the model system does not respond to known triggers of reactivation under hypoxic conditions suggests that hypoxia may affect the RTA driven expression of DsRed from the PAN lytic promoter. This could be for two major reasons; firstly translational arrest is induced by hypoxia (see section 1.5.2). This is a complex process which consists of two effector pathways; the first is via activation of PERK, an ER kinase, which phosphorylates and inactivates the translation initiation factor, eIF2α, blocking translation (Koumenis et al., 2002). The second is via disruption of the eIF4F cap-binding protein complex after prolonged hypoxic exposure (Koritzinsky et al., 2006). Therefore RTA and/or DsRed translation may be blocked under hypoxic conditions.

The second explanation, for the absence of DsRed in response to hypoxia, is the requirement of molecular oxygen in the final maturation stage of fluorescent proteins. DsRed initially undergoes the same maturation process as GFP; the three steps are cyclisation of the main chain, dehydration and finally oxidation to form a fully mature fluorescent protein (Heim et al., 1994). However, DsRed requires a further oxidation step responsible for the red shift in fluorescence (Gross et al., 2000). Therefore, DsRed requires two oxygen dependent steps to fluoresce and is particularly sensitive to hypoxia. Heim *et.al*. demonstrated that fluorescence gradually developed when oxygen was introduced to GFP-expressing bacteria grown anaerobically, importantly, this was independent of new protein synthesis (Heim et al., 1994). Further to this, Gross *et.al.* showed

that the conversion of green fluorescent DsRed to red fluorescent DsRed requires molecular oxygen (Gross et al., 2000). Therefore, under hypoxic conditions, even if RTA and consequently DsRed protein were synthesised, fluorescence may not occur due to the absence of oxygen and a failure of the chromophore to mature.

We favour oxygen dependent fluorescence as the explanation as to why DsRed expression is not seen in Clone 9 cells after 48 hours hypoxic treatment; as returning hypoxic cultures to oxygen conditions (reoxygenation) resulted in rapid DsRed expression (Figure 4-5 and Figure 4-7). Weak DsRed expression is seen in Clone 9 and r219.KSHV JSC-1 cells after 72 hours hypoxia treatment indicating hypoxic induction of DsRed expression but the lack of sufficient oxygen to allow maturation of all the fluorescent protein produced (Figure 4-5 and Figure 4-7). The presence of RTA protein in cells under hypoxia but not under normoxic conditions argues against translational arrest of RTA and DsRed protein production, although we did not assay for DsRed protein production (Figure 4-5). Together this data shows that the conditions established do represent true hypoxic conditions that lead to KSHV reactivation, but this could and should not be monitored by PAN specific DsRed expression. Further study therefore focused on the presence of RTA as a measure of reactivation. Results also show that the length of hypoxic exposure time is important as XBP-1s, HIF-1α and RTA are robustly upregulated at 72 hours and not at 24 (Figure 4-5 and Figure 4-8).

Experiments were carried out using rKSHV.219 HEK 293T cells, Clone 9, and a range of PEL cell lines in order to address the role of HIF-1α and XBP-1s in KSHV reactivation. Previous studies displaying the ability of HIF-1α to activate transcription from ORF50 promoter-reporters show cell type specific results. HIF-1α overexpression under normoxia in HEK 293 cells was shown to increase RTA promoter activity substantially more than in BJAB cells (Cai et al., 2006a). In contrast overexpression of HIF-1α in Hep3B cells only weakly induced the ORF50 promoter and RTA expression in the absence of hypoxia (Haque et al., 2003), a situation more akin to that found in KSHV infected endothelial cells (Carroll et al., 2006). Indeed, overexpression of HIF-1α under normoxia in PEL cell lines failed to induce RTA expression, whereas XBP1s overexpression

efficiently initiates the KSHV lytic cycle (Figure 4-13 and Figure 4-14) (Wilson et al., 2007). In contrast HIF-1α was able to induce RTA promoter activity under normoxia in HEK 293T cells but in the absence of virus (Figure 4-10). Together this suggests a role for KSHV in maintaining latency through the control or suppression of HIF-1α activity on the ORF50 promoter under normoxia.

Under normoxic culture conditions KSHV LANA mediates degradation of the Von Hippel-Lindau factor (VHL), the master regulator of HIF stability, thereby stabilising HIF-1α upon KSHV infection (Cai et al., 2006b). HIF-1α mRNA is induced in KSHV infected endothelial cells and HIF-1α protein is abundantly expressed in KS lesions, where it is nuclear localised (Carroll et al., 2006). HIF-1α induces the upregulation of cytokines for neoangiogenesis and the growth of target endothelial cells and is therefore able to contribute to the high vascularisation and location of KS lesions in the relatively hypoxic environment of the skin (Bedogni et al., 2005), particularly on the lower extremities of those with poor circulation, the elderly and diabetes patients (Ensoli et al., 2001a) (Laor and Schwartz, 1979). LANA, via its N-terminal domain also mediates HIF-1α nuclear accumulation in PEL cell lines (Cai et al., 2007). Additionally, B-cells are exposed to low oxygen tensions as they develop in secondary lymphoid tissues and migrate between the blood and different tissues, possibly contributing to the HIF-1α accumulation in PEL (Caldwell et al., 2001).

Despite the nuclear accumulation and stabilisation of HIF-1α in PEL, infected endothelial cells and KS lesions very little KSHV lytic replication is observed, typical of the infected cells being predominantly latent. The nuclear accumulation of HIF-1α in response to KSHV latent infection and hypoxia are spatially distinct. This suggests that accumulation in nuclear compartments under normoxia does not result in the induction of RTA and that redistribution of HIF-1α under hypoxia is required for the induction of the lytic cycle (Cai et al., 2007) (Zheng et al., 2006). Alternatively, KSHV associated HIF-1α accumulation could prime infected cells so that when exposed to hypoxia the nuclear stabilised HIF-1α is further activated, for example by phosphorylation (Richard et al., 1999), resulting in lytic reactivation (Carroll et al., 2006). This ‘superinduction’ of HIF-1α in response to hypoxia has been observed through the increased activity of HIF-1α by v-SRC (Jiang et al., 1997a). Interestingly, not

all studies, including ours, are able to visualise stabilised HIF-1α consistently in normoxic PEL; suggesting that levels may be very low or transient and therefore insufficient for transactivation. However, VEGF, a HIF-1α responsive gene is upregulated in a KSHV LANA-induced, HIF-1α dependent way under normoxia (Carroll et al., 2006) (Cai et al., 2007) indicating that HIF-1α can be transcriptionally active under latent normoxic infection. It is possible an additional factor(s) activated by hypoxia and absent during latent KSHV infection are necessary for HIF-1α mediated lytic induction. Overexpression of XBP-1s in conjunction with HIF-1α under normoxia resulted in no further accumulation of RTA over and above that seen with XBP-1s alone (Figure 4-14), indicating that XBP-1s is not the additional factor required by HIF-1α to induce KSHV lytic cycle under hypoxia.

It is likely that the architecture of the hypoxia-induced transcriptional complex assembled on the RTA promoter contains other factors. High cell density, through nutritional starvation, can lead to the upregulation of hypoxia-inducible genes, for example carbonic anhydrase 9 (CA9), in a HIF-1α independent way under normoxia and an enhanced upregulation in a HIF-1α dependent way under hypoxia; an effect mediated by the transcription factor Ets-1 (Salnikow et al., 2008). Ets-1 has been reported to induce KSHV lytic replication directly and is important for TPA-induced reactivation (Yu et al., 2007b). Ets-1 and other Ets family members have also been identified as potential coactivators of XBP-1s leading to the possibility of an HIF-1α, Ets, XBP-1 complex being formed on the ORF50 promoter (Acosta-Alvear et al., 2007). HIF-1α responsive genes such as VEGF and GLUT-1 can also be induced differentially by hypoglycaemia, a metabolic insult that often occurs in parallel but also independently of hypoxia (Stein et al., 1995). Further, it has been documented that the HIF-1α binding site consists of the HRE as well as the HIF-1α ancillary sequence (HAS), a required cis-acting element for transcriptional activation under hypoxia (Forsythe et al., 1996). Also, HAS binding factors may be distinct for VEGF compared to other hypoxia inducible genes (Kimura et al., 2001).

PEL cell lines have minimal KSHV lytic induction and contain inactive XBP-1u and nuclear HIF-1α, both of which can be rapidly activated by relevant stresses leading to lytic replication (Wilson et al., 2007) (Cai et al., 2006b). Why XBP-1

is inactive and nuclear accumulation of HIF-1α fails to induce the KSHV lytic cycle in PEL cells is not known. However, both transcription factors play important and varied roles at different stages of normal B-cell differentiation. XBP-1s, as part of the physiological UPR is critical for plasma cell differentiation (Reimold et al., 1996) (Reimold et al., 2001) (Iwakoshi et al., 2003). In an analogous way B-cells also experience hypoxic microenvironments and HIF-1α stabilisation in normal physiology (Caldwell et al., 2001). HIF-1α is expressed in the microenvironments of the germinal centres (GCs) (Piovan et al., 2007), secondary lymphoid tissues and bone marrow (Parmar et al., 2007). In the GC environment HIF-1α has been shown to upregulate the chemokine receptor CXCR4 and its ligand CXCL12 (Ceradini et al., 2004). This is required for the correct homing of a circulating normal B-cell and can lead to metastasis in malignant cases. In fact PEL express CXCR4 on their surface and home to the CXCL12 expressing mesothelium; possibly giving rise to the body cavity based presentation of PEL (Foussat et al., 2001). Interestingly, XBP-1s has also been shown to be induced by B-cell receptor (BCR) cross linking; an event that occurs within the GC (Skalet et al., 2005). Additionally, HIF-1α deficient chimeric mice display a distortion in the number of B-2 lymphocytes in the bone marrow as well as an accumulation of peritoneal B-1 cells and autoimmunity (Kojima et al., 2002). This places HIF-1α and XBP-1s with a role in normal B-cell biology as well as being usurped in reactivation of KSHV through shared response elements in the ORF50 promoter. It is possible that antigen stimulated cells of the HIF-1α-positive germinal centre provide the cues for the KSHV lytic cycle induction in primary infection; whereas terminal differentiation of B-cells to plasma cells at the activation of XBP-1 provide the lytic cues during life-long persistent oral shedding.

The co-involvement of HIF-1α and XBP-1s may also be of significance in other herpesviruses. Epstein Barr virus (EBV), the most closely related human herpesvirus to KSHV, is also induced into its lytic cycle by hypoxic treatment (Figure 4-15) (Jiang et al., 2006). XBP-1s has also been implicated as an EBV lytic cycle inducer, although most likely in the presence of other co-activators such as protein kinase D (Bhende et al., 2007) (Sun and Thorley-Lawson, 2007) (McDonald et al., 2010). In the murine herpesvirus 4 (MuHV-4), a related Rhadinovirus, ORF50 is also upregulated in response to hypoxia (Polcicova et

al., 2008) although a response to XBP-1s has not been reported. Similar to KSHV LANA, the EBV latent membrane protein 1 (LMP-1) has been reported to upregulate HIF-1α (Kondo et al., 2006; Wakisaka et al., 2004). Together, these data suggest that gamma herpesviruses have evolved the ability to respond to cellular stress transcription factors and that these same factors are crucial for normal B-cell development, where their expression during differentiation may facilitate controlled KSHV lytic cycle activation.

5 Separating plasma cell differentiation from KSHV reactivation

5.1 Introduction

The interactions between virus and host are numerous and complex; however, certain cellular pathways are often manipulated by many viruses (Fossum et al., 2009). These key pathways are crucial, usually enabling the virus to replicate and persist. The UPR is an example of a host response targeted by several viruses (see section 1.3). The manufacture of viral proteins increases the load on the host folding apparatus triggering ER-stress pathways. The ways in which viruses modify the UPR are not completely understood; in some cases viruses encode proteins to act directly for example, the human cytomegalovirus US11 protein (He, 2006).

The data in chapter 3 shows that KSHV reactivation is directly linked to the UPR via XBP-1s, and in chapter 4 we show that KSHV can also reactivate in response to hypoxia, another ER-stress inducing condition. Whether KSHV modulates the UPR during reactivation is unclear; however, from gene expression profiling of PEL cells latently infected with KSHV we can see that the UPR is only partially active in these cells (Jenner et al., 2003) (Klein et al., 2003). This profile identifies PEL cells as plasmablast-like, representing a late stage of B-cell differentiation prior to plasma cells, having active ATF6 and unspliced XBP-1 (Jenner et al., 2003) (see sections 1.1.8.2 and 1.4.4). However, IRE-1 is functional in PEL cells (Wilson et al., 2007) and therefore it is not clear whether another UPR defect, insufficient ER stress (van Anken et al., 2003) or latent KSHV is responsible for the lack of active XBP-1.

As previously introduced, XBP-1 is essential for plasma cell differentiation to facilitate the production of vast amounts of antibody (Reimold et al., 2001) (Iwakoshi et al., 2003) (see section 1.4.4). PEL cells do not secrete immunoglobulin and many do not express surface immunoglobulin (Nador et al., 1996). Supplying XBP-1s to PEL cells results in KSHV reactivation and importantly, the induction of gene expression changes relating to plasma cell

differentiation (Wilson et al., 2007) (Shaffer et al., 2004). The UPR that occurs during B-cell terminal differentiation or indeed the differentiation of any professional secretory cell can be termed 'physiological UPR' (Iwakoshi et al., 2003) (see section 1.4.4). This specific UPR may be comprised of one or more branches of the pathway. However, the cellular changes involved provide the ideal environment of an expanded secretory system in the absence of translational arrest, for the production of herpes virions. KSHV reactivation and B-cell differentiation are therefore firmly connected.

The aim of this chapter was therefore two-fold: firstly, to further understand the interactions between KSHV and its host cell during XBP-1s-induced reactivation and secondly, to explore the final stages of B-cell development, as PEL cells provide the correct transcriptional environment i.e. BCL-6^- and BLIMP-1^+ for this. However, plasma cell differentiation requires XBP-1s and we have established that this transcription factor leads to KSHV reactivation. We therefore needed to remove the impact of the reactivating virus in order to observe the changes that occur solely during plasma cell differentiation. This we attempted by preventing the reactivation of KSHV and supplying XBP-1s to PEL cells. KSHV lytic replication is dependent on the presence of the immediate early, RTA protein (Lukac et al., 1999) (Xu et al., 2005), in this chapter we therefore employ both shRNA and dominant negative techniques to target RTA and prevent KSHV reactivation.

Gene expression changes resulting from XBP-1s expression in PEL cells that are unable to reactivate should represent changes that occur during the terminal differentiation of B-cells. Removing these differentially expressed genes from the changes seen when XBP-1s is supplied to PEL cells, with no block to reactivation, should provide information about the virus-host interface.

5.2 Results

5.2.1 Characterisation of shRNA-RTA-expressing JSC-1 50P-2 cell line

A short hairpin RNA was previously designed, by Sam Wilson, to target the 3' region of RTA (Figure 5-1 B) and cloned into a lentivirus backbone. JSC-1 cells were transduced and selected, to generate stable shRNA-RTA-expressing clonal cell lines. RTA expression in response to TPA treatment of these cell lines was measured by staining and FACs analysis. The cell line JSC-1 50P-2 was determined to have the lowest number of cells expressing RTA. Consistent with the accepted role of KSHV RTA, the block prevented subsequent lytic gene expression and virion production (Sam Wilson – unpublished data).

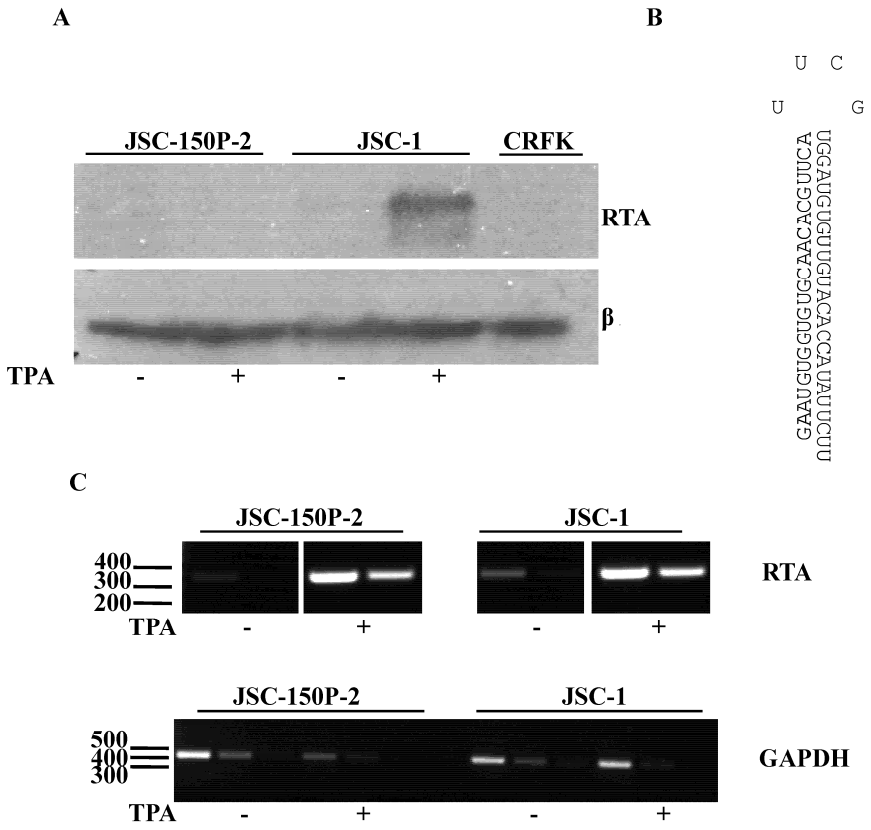


Figure 5-1 Characterisation of shRNA-RTA-expressing JSC-1 50P-2 cell line
(A) Western blot analysis of whole cell lysates of the KSHV negative cell line CRFK and the PEL cell lines, parental JSC-1 and JSC-1 stably expressing shRNA-RTA (JSC-1 50P-2); treated or untreated with 20 ng/µl TPA. Samples taken 48 hours after treatment are blotted with polyclonal rabbit anti-RTA. RTA is induced by TPA treatment of the parental JSC-1 cells only. β-actin acts as a loading control. (B) Sequence and probable structure of the shRNA targeting the 3' region (73546 – 73569) of RTA mRNA. Numbering corresponds to KSHV genomic sequence (gi: 18845965). (C) RT PCR amplification of RTA from the total mRNA of parental JSC-1 and JSC-1 50P2 cells, untreated or treated with TPA. Two ten-fold dilutions of cDNA are shown for each sample. Similar levels of RTA transcript are seen in the parental and shRNA-expressing JSC-1 samples. RT PCR for GAPDH on the same RNA was used to normalise cDNA input and three ten-fold dilutions of cDNA are shown for each sample.

To ensure that shRNA-RTA expression is maintained, JSC-1 50P-2 cells were thawed and cultured under puromycin selection for one week. Parental JSC-1 or JSC-1 50P-2 cells were then cultured in the presence of 20 ng/µl TPA overnight to induce the KSHV lytic cycle. 48 hours after treatment RTA expression was monitored by RT PCR and western blot analysis. No RTA protein was detected by western blot in the untreated or TPA treated JSC-1 50P-2 cell line samples (Figure 5-1 A). In comparison an increase in RTA expression was seen in the parental JSC-1 cells in response to the lytic cycle inducer TPA, compared to untreated controls (Figure 5-1 A). However, semi-quantitative RT PCR showed similar levels of RTA transcript in the TPA treated samples of both the parental and shRNA-RTA-expressing cell lines (Figure 5-1 C). This data indicates that shRNA-RTA expression is maintained in the JSC-1 50P-2 cells and acts to block RTA translation rather than induce transcript degradation.

5.2.2 Exogenous XBP-1s induces KSHV lytic cycle in the presence of shRNA-RTA

We have shown that KSHV reactivation and plasma cell differentiation are linked by the transcription factor XBP-1s; see chapter 3 (Wilson et al., 2007). We wanted to ascertain the interactions between virus and host when XBP-1s is supplied to PEL cells. In order to separate differentiation from reactivation we attempted to prevent XBP-1s-induced reactivation with shRNA targeting RTA. We, therefore, transduced shRNA-RTA-expressing JSC-1 50P-2 cells or the parental JSC-1 cells with XBP-1s-expressing lentivirus or empty control at an input equivalent to an MOI 5 on HEK 293-T cells. TPA treatment was performed, as above, as a control and the resulting KSHV lytic replication in each sample was monitored 48 hours later. RTA protein was expressed in both the TPA treated and XBP-1s-expressing JSC-1 parental cell samples as expected (Figure 5-2 A). As in section 5.2.1, RTA protein was not seen in TPA treated JSC-1 50P-2 cells; however, a weak band corresponding to RTA could be seen in the XBP-1s-expressing sample (Figure 5-2 A). The level of overexpressed XBP-1s in the parental cell line was similar to that of the shRNA-RTA-expressing cell line, as determined by western blot (Figure 5-2 A). RTA protein was not detected in either cell line in the non-transduced samples, or samples transduced with empty vector control (Figure 5-2 A).

To confirm that the absence of RTA protein does prevent KSHV lytic cycle induction we analysed the expression of a spliced early lytic gene ORF29a/b by RT PCR. ORF29a/b transcript was present at low levels in parental JSC-1 cells transduced with empty vector control (Figure 5-2 B). However, far higher levels of ORF29a/b mRNA were detected in the parental JSC-1 samples treated with TPA or transduced with XBP-1s (Figure 5-2 B). In the JSC-1 50P-2 samples ORF29a/b was only amplified from the sample transduced with XBP-1s (Figure 5-2 B). This suggests that when RTA is absent in TPA treated JSC-1 50P-2 cells KSHV lytic replication is not induced. Unfortunately, the shRNA-RTA expressed by the stable JSC-1 50P-2 cell line is not able to prevent reactivation in all cells in response to exogenous XBP-1s.

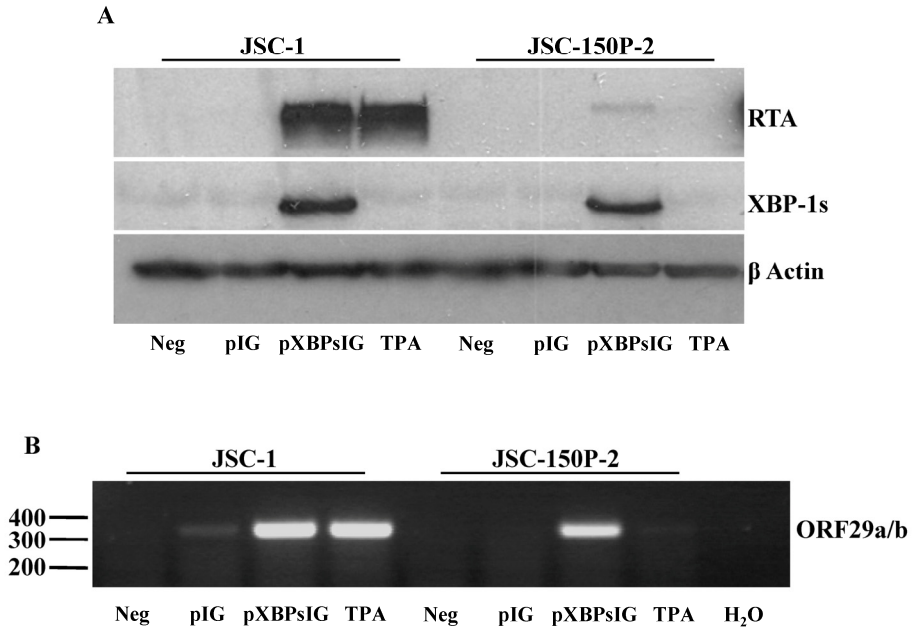


Figure 5-2 JSC-1 50P-2 express RTA in response to exogenous XBP-1s
(A) Whole cell lysate anti-RTA and XBP-1s western blot of parental JSC-1 cells and JSC-1 stably expressing shRNA-RTA (JSC-1 50P-2) either non-transduced (Neg), transduced with empty vector (pIG) or XBP-1s-expressing vector (pXBPsIG) at an input equivalent to an MOI 5 on HEK 293-T cells or treated with TPA. RTA is induced in the parental JSC-1 cells after TPA treatment and in XBP-1s-expressing samples. Weak RTA expression is detected only in the JSC-1 50P-2 sample transduced with XBP-1s. XBP-1s is expressed to similar levels in both cell lines transduced with pXBPsIG. β-actin acts as a loading control. (B) Total cellular RNA was isolated from parallel cultures of the samples in A. RT PCR and gene specific primers for the spliced lytic transcript, ORF29a/b, were used to amplify a 300bp product from cDNA. PCR product is seen in both TPA treated and XBP-1s-expressing parental JSC-1 samples. A slightly reduced level of ORF29a/b transcript is also seen in the JSC-1 50P-2 cells containing shRNA-RTA transduced with XBP-1s. RT PCR for β-actin on the same RNA was used to normalise cDNA input.

5.2.3 DTT treatment causes splicing of endogenous XBP-1 and cell death in JSC-1 cells

It has previously been shown that DTT treatment of PEL cells causes XBP-1 splicing (Wilson et al., 2007). We wanted to determine whether this endogenous level of XBP-1s would induce KSHV reactivation in PEL, which could then be blocked by shRNA targeted to RTA. We therefore cultured parental JSC-1 and the shRNA-RTA-expressing JSC-1 50P-2 in the presence of 3 mM DTT for 1 hour. At this point samples were taken to monitor XBP-1s splice status by RT PCR. Similar levels of increased XBP-1 spliced transcript were present in both the parental JSC-1 and the JSC-1 50P-2 cell lines compared to untreated controls (Figure 5-3 A). 48 hours after the 1 hour DTT treatment, samples were taken to monitor KSHV reactivation by RT PCR for the spliced lytic transcript of ORF29a/b. DTT led to significant cell death 4-6 hours after treatment of both the parental JSC-1 and the JSC-1 50P-2 cell lines. Lytic transcript was therefore only detected in the positive control TPA treated sample of the parental JSC-1 cell line (Figure 5-3 B). DTT therefore cannot be used to investigate the ability of endogenous XBP-1s to activate KSHV RTA expression in response to ER-stress in JSC-1 cells. Therefore, we attempted to remove the residual RTA activity in JSC-1 50P2 cells.

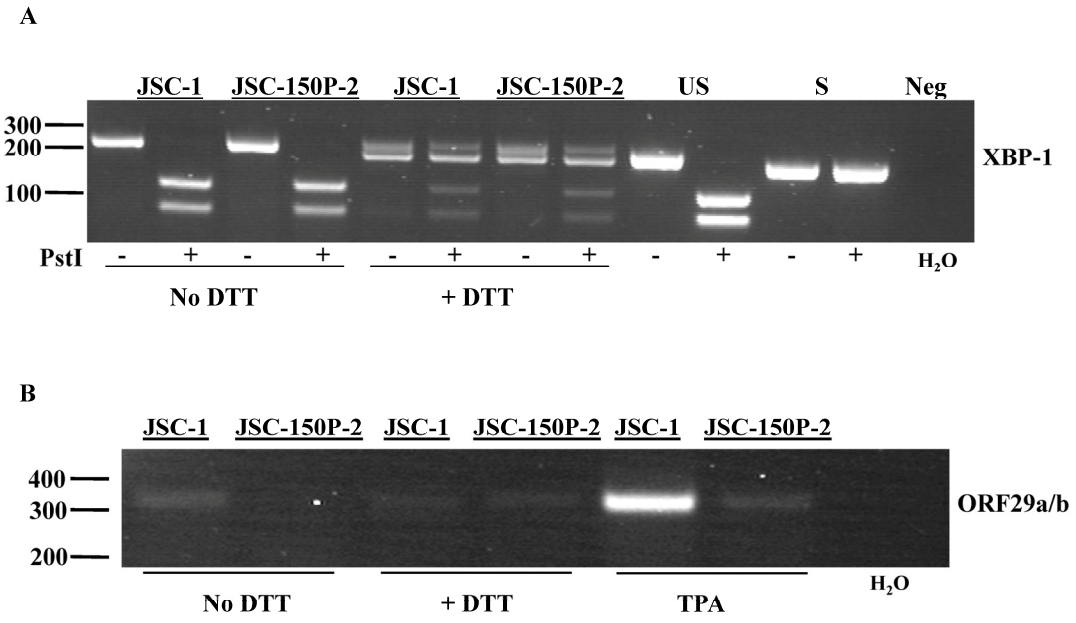


Figure 5-3 JSC-1 cell response to DTT
(A) RT PCR amplification across the XBP-1 intron produces a 249 base pair amplicon from XBP-1u mRNA and a 223 base amplicon from XBP-s mRNA. Pst I digests only the XBP-1u amplicon resulting in two bands, whereas XBP-1s results in a single band (Wilson et al., 2007). RT PCR amplification from the total mRNA parental JSC-1 cells and JSC-1 stably expressing shRNA-RTA (JSC-1 50P-2) treated or untreated with 3 mM DTT shows that XBP-1s is produced

after 1 hour treatment. XBP-1 remains unspliced in samples not treated with DTT. In the DTT treated samples a slower-migrating non-Pst I digestible PCR hybrid between the XBP-1s and XBP-1u products is visible similar to previously described (Wilson et al., 2007). β-actin on the same RNA was used to normalise cDNA input (data not shown). (B) A 300bp product was amplified from RNA samples taken 48 hours after DTT treatment with gene specific primers for the spliced lytic transcript, ORF29a/b. PCR product is seen only in the parental JSC-1 samples after TPA treatment. ORF29a/b transcript is not seen in the DTT treated XBP-1s-positive samples as this treatment was toxic after 4-6 hours. RT PCR for β actin on the same RNA was used to normalise cDNA input (data not shown).

5.2.4 Generating a dominant negative form of RTA

The KSHV RTA protein contains a potent carboxy-terminal transactivation domain (Figure 5-4 A). A truncated mutant created by deletion of the C-terminal 161 amino acids of RTA, does not cause transcriptional activation (Lukac et al., 1999). Through multimerisation with wild-type RTA the C-terminally truncated RTA functions as an RTA specific dominant negative inhibitor of transactivation (Bu et al., 2007). Since shRNA targeted to RTA was unable to completely block KSHV reactivation induced by ectopic XBP-1s we wanted to determine if a dominant negative RTA could be used instead. We therefore designed primers to PCR the first 530 amino acids of RTA, and that would add a nuclear localisation signal to the C-terminus of these amino acids (Figure 5-4 B). PCR amplification, using wild-type RTA as a template, provided a truncated fragment. This was cloned into a HA-tagged lentivirus vector (see materials and methods 2.4.5.6) and transduced into HEK 293T cells where it was detectably expressed at a lower molecular weight to full-length RTA (data not shown).

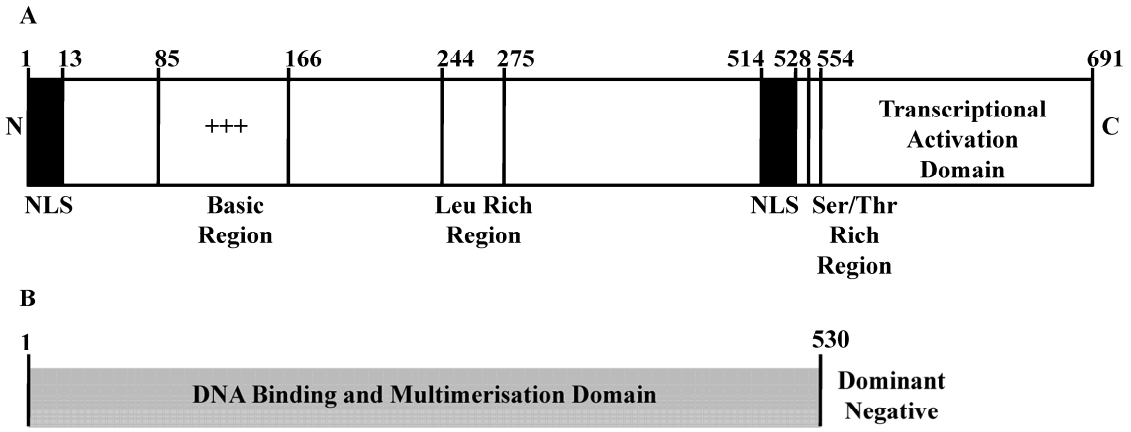


Figure 5-4 Generating a dominant negative form of RTA
(A) Domain map of full-length ORF50/RTA, depicting structural and functional regions. Numbering refers to amino acid positions. Abbreviations: NLS, Nuclear localisation sequence. (B) Deletion of the RTA activation domain results in a dominant negative. Schematic representing the carboxy-terminal truncated dominant negative RTA.

5.2.4.1 Dominant negative RTA can reduce transactivation of the RTA promoter by full-length RTA

We used a luciferase reporter assay to examine the ability of the mutant, dominant negative RTA to interfere with the activity of wild-type RTA. A full-length RTA promoter luciferase construct (p50LUC) was co-transfected into HeLa cells with equal amounts of either wild-type RTA alone or in combination with dominant negative RTA- or a GFP-expressing control plasmid. The luciferase activity obtained from transactivation by wild-type RTA alone was taken as 100 % activation of the system (Figure 5-5). Co-expression of a control plasmid in addition to wild-type RTA led to a slight reduction in this activity (Figure 5-5). However, expression of dominant negative RTA with wild-type RTA reduced luciferase activity by around 50 % (Figure 5-5). Therefore, the C-terminal truncated RTA mutant generated inhibits the transactivation ability of wild-type RTA when expressed at the same time as full-length RTA and in equal amounts.

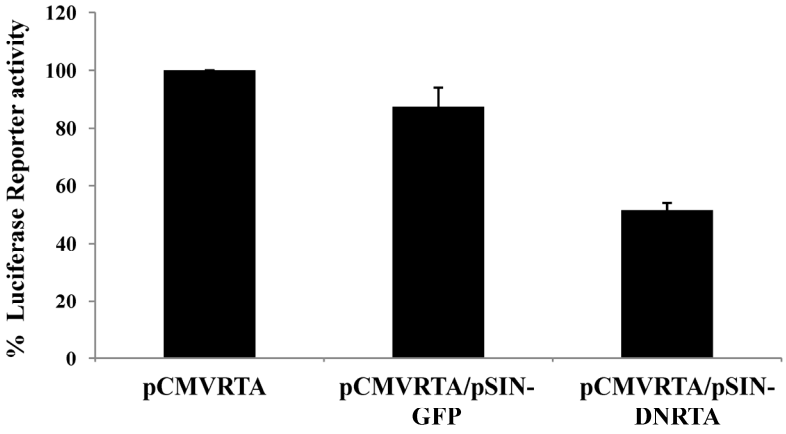


Figure 5-5 Dominant negative RTA activity
HeLa cells were transfected with a *Renilla*-expressing control plasmid (phRL-null) plus the ORF50 promoter *Firefly* luciferase reporter plasmid (p50LUC) with either wild-type RTA (pCMVRTA) alone or in combination with vector expressing GFP control (pSIN-GFP) or expressing dominant negative RTA (pSIN-DNRTA). *Firefly* luciferase activity was determined 48 hours post-transfection and normalised to *Renilla* luciferase (phRL-null) activity. Wild-type RTA was able to transactivate the RTA promoter and luciferase expression. The activity of full-length RTA on the promoter was plotted as 100%. GFP co-expression reduced the activity of full-length RTA minimally. However, the presence of dominant negative RTA reduced this activity to approximately 50 % ($p<0.05$ 95% CFI). Columns represent the mean of three independent experiments and error bars represent standard error of the mean.

5.2.4.2 Dominant negative RTA is unable to block DTT-induced rKSHV.219 reactivation in Clone 5 cells

To test the ability of dominant negative RTA to block the activity of endogenous RTA we used rKSHV.219 HEK 293T, Clone 5 cells. We have previously shown

that DTT treatment of these cells induces XBP-1s and KSHV reactivation which is dependent on RTA; see section 3.2.5. We therefore transfected Clone 5 cells with 8 µg of lentivirus vector expressing dominant negative RTA- or GFP-expressing control. After 48 hours cells were treated with 3 mM DTT or remained untreated overnight. Following a further 48 hours, cells were sampled for western blot and RT PCR analysis of the KSHV lytic cycle. The level of DsRed-positive cells increased in all DTT treated samples to similar levels (data not shown). Full-length RTA protein was detectable in all Clone 5 cell samples treated with DTT, and was not seen in untreated samples (Figure 5-6 A). Similar levels of dominant negative RTA were present in both untreated and DTT treated samples (Figure 5-6 A).

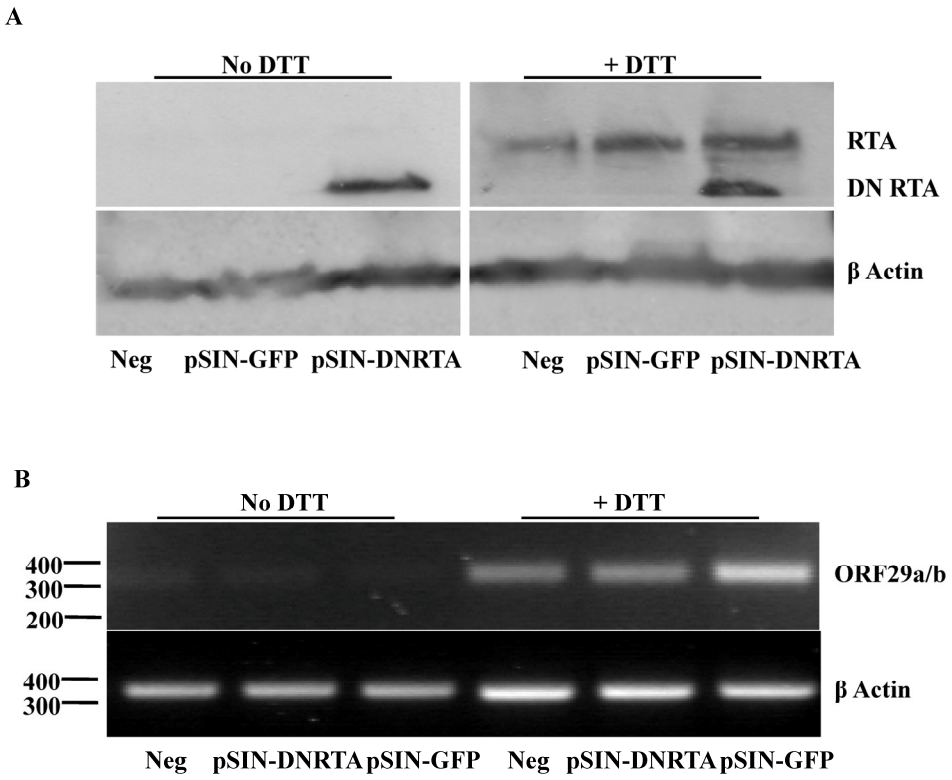


Figure 5-6 DTT treatment of Clone 5 cells expressing dominant negative RTA
(A) Anti-RTA western blot of the Clone 5 whole cell lysates either untransfected (Neg), transfected with 8 µg GFP-expressing vector (pSIN-GFP) or 8 µg dominant negative expressing vector (pSIN-DNRTA); left untreated or treated overnight with 3 mM DTT. Full-length RTA protein was detected only in DTT treated samples indicating KSHV reactivation in these samples. The expression of dominant negative RTA can be seen running at a lower molecular weight than full-length RTA in all samples transfected with pSIN- DNRTA. β-actin acts as a loading control. (B) Total cellular RNA was isolated from parallel cultures of the samples in A. RT PCR and gene specific primers for the spliced lytic transcript, ORF29a/b, were used to amplify a 300bp product from cDNA. PCR product is seen in all DTT treated Clone 5 cell samples including the sample expressing dominant negative RTA. RT PCR for β-actin on the same RNA was used to normalise cDNA input.

If the dominant negative is acting to inhibit the action of RTA then the KSHV lytic cycle should be blocked. We therefore looked by RT PCR for the spliced lytic transcript ORF29a/b after DTT treatment of Clone 5 cells in the presence of dominant negative RTA. The semi-quantitative PCR revealed similar levels of lytic transcript in the untransfected and dominant negative RTA transfected samples (Figure 5-6 B). Slightly higher levels of ORF29a/b were seen in the GFP-expressing Clone 5 cells (Figure 5-6 B). These results suggest that overexpression of dominant negative RTA is not able to block KSHV reactivation in response to DTT in rKSHV.219 HEK 293T cells.

5.2.4.3 Exogenous XBP-1s induces KSHV lytic cycle in JSC-1 expressing dominant negative RTA

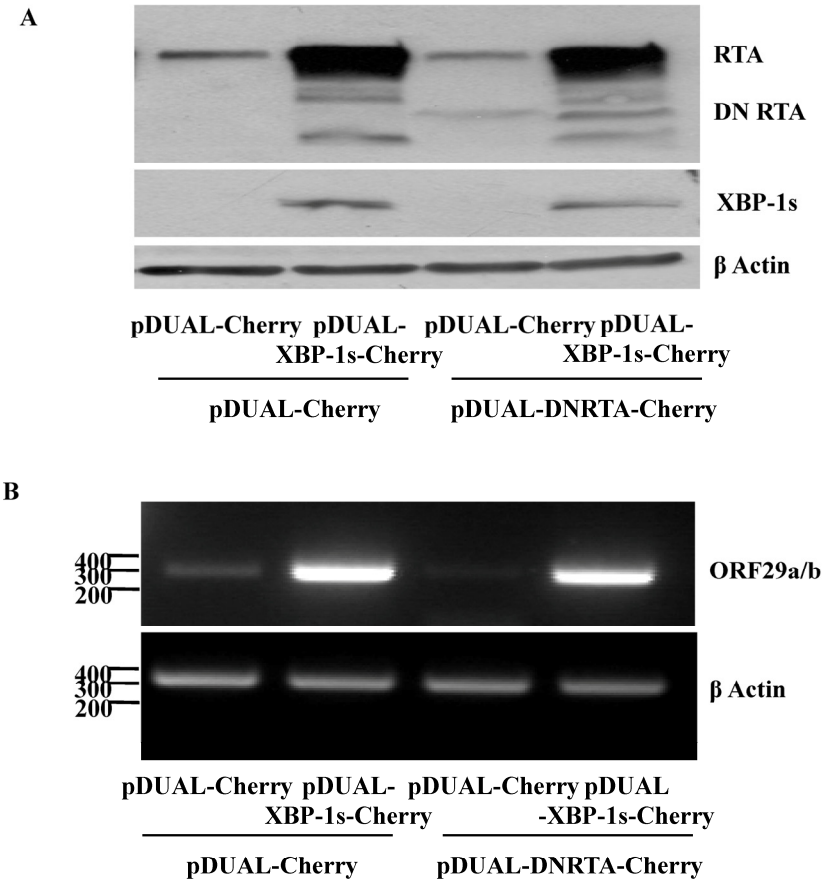


Figure 5-7 XBP-1s overexpression in JSC-1 cells expressing dominant negative RTA
(A) Whole cell lysate anti-RTA and XBP-1s western blot of JSC-1 cells either transduced with empty vector (pDUAL-Cherry) or dominant negative-expressing vector (pDUAL-DNRTA-Cherry) at an input equivalent to a MOI 5 on HEK 293-T cells, in combination with either empty vector (pDUAL-Cherry) or XBP-1s-expressing vector (pDUAL-XBP-1s-Cherry) at an input equivalent to a MOI 2 on HEK 293-T cells. RTA is induced in the XBP-1s-expressing samples regardless of the presence of dominant negative RTA, which can be seen at a lower molecular weight. β-actin acts as a loading control. (B) Total cellular RNA was isolated from parallel cultures of the samples in A. RT PCR and gene specific primers for the spliced lytic transcript, ORF29a/b, were used to amplify a 300bp product from cDNA. A feint band of ORF29a/b transcript is also seen

in the samples transduced with pDUAL-Cherry. However, a strong PCR product is seen in both XBP-1s-expressing JSC-1 samples including cells expressing dominant negative RTA. RT PCR for β-actin on the same RNA was used to normalise cDNA input.

We wanted to assess the ability of dominant negative RTA to prevent reactivation in the PEL cell model. JSC-1 cells were transduced with an empty or dominant negative RTA-expressing lentivirus at an input equivalent to an MOI 5 on HEK 293-T cells, in combination with either empty or XBP-1s-expressing lentivirus at an input equivalent to a MOI 2 on HEK 293-T cells. After 48 hours western blot analysis confirmed that ectopic expression of XBP-1s in JSC-1 cells induces RTA expression in the presence or absence of dominant negative RTA (Figure 5-7 A). It should be noted however that the dominant negative RTA was expressed at lower levels in JSC-1 cells.

RT PCR for the lytic gene ORF29a/b revealed transcript at levels comparable to TPA-induced reactivation in all samples expressing XBP-1s (Figure 5-7 B and data not shown). The dominant negative RTA generated by truncation of the full-length RTA, is unable to inhibit the reactivation caused by the presence of exogenous XBP-1s in JSC-1 cells.

5.2.5 Separating plasma cell differentiation from KSHV reactivation using gene expression profiling

We have so far determined that although shRNA-RTA can effectively block KSHV reactivation by the known inducer TPA, it cannot stop the induction of the lytic cycle by XBP-1s. The dominant negative RTA generated can inhibit the transactivation of the ORF50 promoter by full-length RTA, but is unable to prevent reactivation in response to XBP-1s. We therefore do not have a method for comparing XBP-1s in PEL cells to PEL cells blocked for RTA activity and KSHV lytic cycle. As an alternative we used a KSHV negative cell line that resembles a post germinal centre stage of B-cell development, DEL, as the control cell line.

Tumours are known to resemble the cell-type from which they arise, which is particularly true of B-cell lymphomas that can be classified by morphology, immunophenotypes and Ig gene sequences (Kuppers et al., 1999) (see section 1.4.5). Recent analysis of the gene expression profiles of a range of B-cell

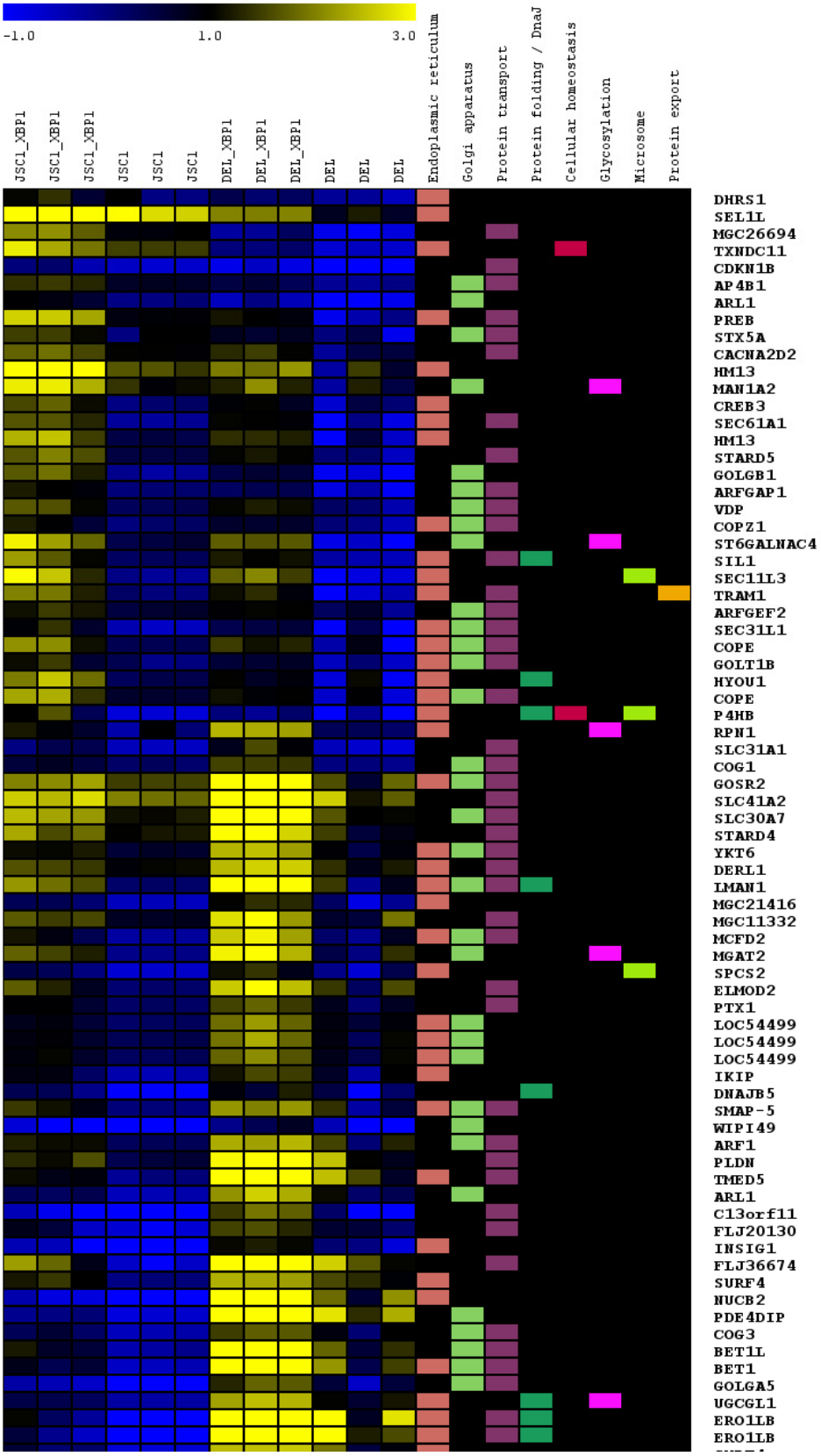
lymphoma cell lines demonstrated clustering that depends on the type of tumour and stage of B-cell development they derive from (Jenner et al., 2003). A major branch point was shown to separate pre-GC and GC-B-cells from post GC derived cells, which is further divided into PEL and plasma cells. One of the shortest tree distances joins DEL cells, in the post-GC branch of this major cluster, to the next branch composed of PEL cells, which are of a plasmablast origin (Jenner et al., 2003). This analysis indicates that the gene expression profile of DEL is closely related to PEL, second only to plasma cells. Therefore, the KSHV negative cell line DEL was used in this experiment as an alternative to preventing lytic replication in JSC-1 cells. Comparison with JSC-1 cells acts as a control for the effect of KSHV because the response of DEL cells should be similar to that of the closely related PEL cells.

$2.5x10^5$ JSC-1 or DEL cells were transduced in triplicate experiments with pDUAL-XBP-1s-Cherry or pDUAL-Cherry lentivirus at an input equivalent to a MOI 8 (as determined on HEK 293-T cells). Previous studies have shown that gene expression profiles of empty vector controls are indistinguishable from that of untransduced controls (Wilson et al., 2007). 6 hours post-transduction the cells were washed once and resuspended in fresh medium. 72 hours post-transduction, the cells were pelleted and resuspended in TRIzol reagent. Total RNA was purified from all samples and the quality and quantity assessed using an Agilent bioanalyzer. From this, mRNA was labelled according to the materials and methods (see section 2.11.2). Labelled cDNA was mixed with Cy3-labelled reference RNA and hybridised to the Agilent Whole Human Genome Oligo Microarrays. A common reference RNA mixture was used to enable comparison across the whole sample set. Array analysis was performed in collaboration with Dan Frampton.

To identify the changes in gene expression in response to XBP-1s expression in JSC-1 and DEL, the M- (expression ratio of sample/reference) and A- (expression level of sample gene) values were calculated for the full dataset of 12 arrays after applying background correction and scale-normalisation using R and BioConductor (Gentleman et al., 2004). Using the BioConductor Limma package a linear model was created using cell type (JSC-1 or DEL) and XBP-1s (overexpressed or empty vector) as factors (Smyth, 2005). By creating

appropriate design and contrast matrices, an empirical Bayes method was used to construct a list of 687 genes found to be significantly differentially expressed. Of these genes 556 were upregulated and 131 were downregulated in response to overexpression of XBP-1s in both cell types (Benjamini-Hochberg adjusted p-value < 0.05) (Figure 5-8). Probes with mean intensities across all samples less than twice the background level were removed ($1/2\log_2 RG < 7$), as they were close to background noise levels.

To identify JSC-1-specific changes in gene expression, a similar analysis was performed using the 6 JSC-1 arrays (3 wild-type samples and 3 expressing-XBP-1s) and a second gene list created. Those genes found in both lists or only the first were cell type independent and classified as 'XBP1-responsive' genes. 366 differentially expressed genes were found solely in the second list and were classified as 'JSC-1-specific' genes. These genes could represent cell type dependent gene expression responses to XBP-1s or changes caused by XBP-1s-induced KSHV lytic replication (Figure 5-9). Both the 'XBP-1 responsive' genes represented by the first list and the remaining 'JSC-1 specific' genes from the second list were split into up- and downregulated genes and analysed for functional enrichment using the online DAVID database, applying a Benjamini-Hochberg adjusted p-value cut-off of 0.05 as before; see section 3.2.6 (Huang da et al., 2009) (Dennis et al., 2003).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JSC1
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DEL_XBP1
DEL_XBP1
DEL_XBP1
DEL
DEL
DEL
Endoplasmic reticulum
Golgi apparatus
Protein transport
Protein folding / DnaJ
Cellular homeostasis
Glycosylation
Microsome
Protein export
DHRS1
SEL1L
MGC26694
TXNDC11
CDKN1B
AP4B1
ARL1
PREB
STX5A
CACNA2D2
HM13
MAN1A2
CREB3
SEC61A1
HM13
STARD5
GOLGB1
ARFGAP1
VDP
COPZ1
ST6GALNAC4
SIL1
SEC11L3
TRAM1
ARFGEF2
SEC31L1
COPE
GOLT1B
HYOU1
COPE
P4HB
RPN1
SLC31A1
COG1
GOSR2
SLC41A2
SLC30A7
STARD4
YKT6
DERL1
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MGC11332
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TMED5
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C13orf11
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INSIG1
FLJ36674
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COG3
BET1L
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GOLGA5
UGCGL1
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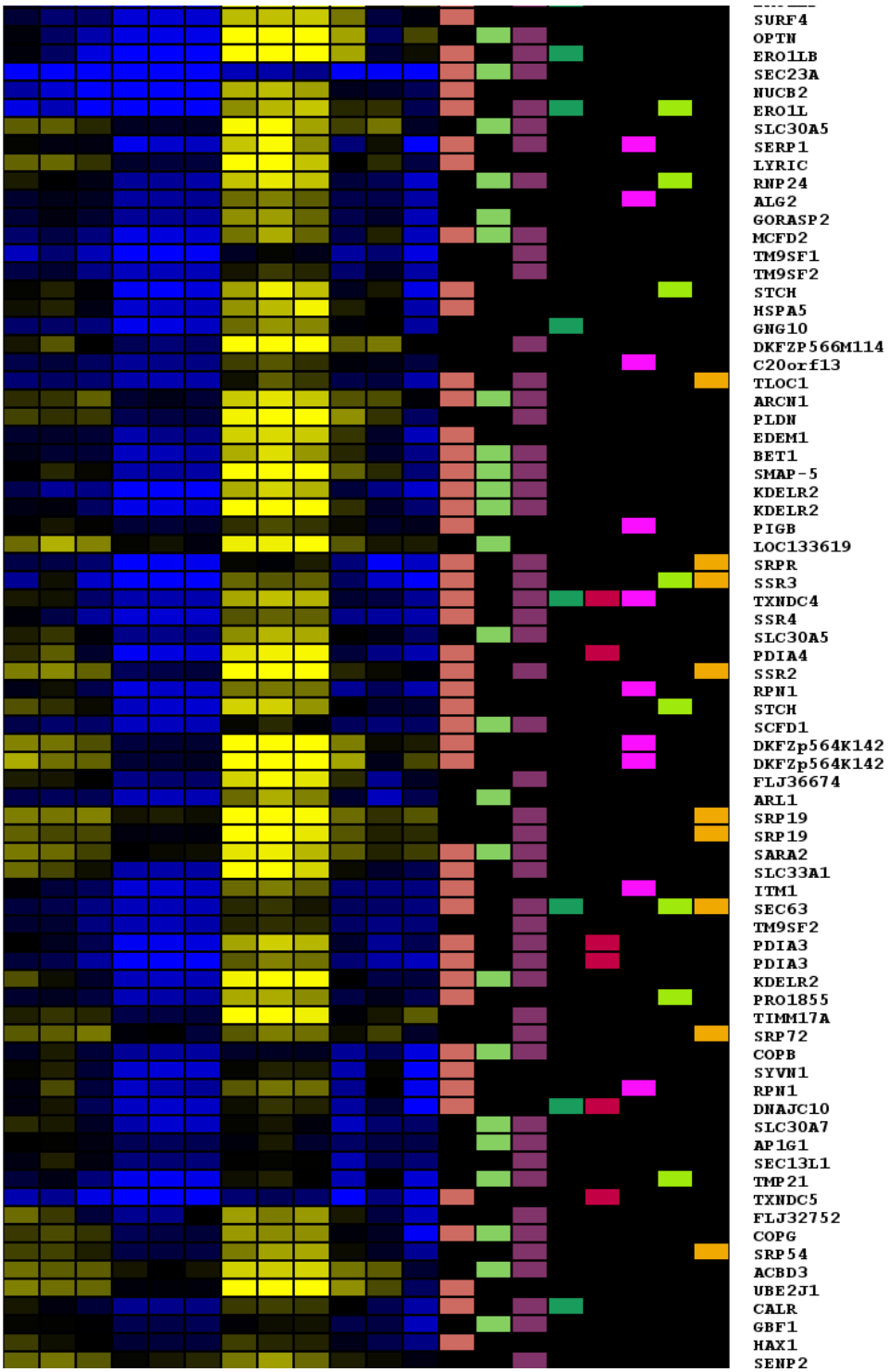
SURF4
OPTN
ERO1LB
SEC23A
NUCB2
ERO1L
SLC30A5
SERP1
LYRIC
RNP24
ALG2
GORASP2
MCFD2
TM9SF1
TM9SF2
STCH
HSPA5
GNG10
DKFZP566M114
C20orf13
TLOC1
ARCN1
PLDN
EDEM1
BET1
SMAP-5
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SLC30A5
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SLC30A7
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SEC13L1
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GBF1
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SENP2

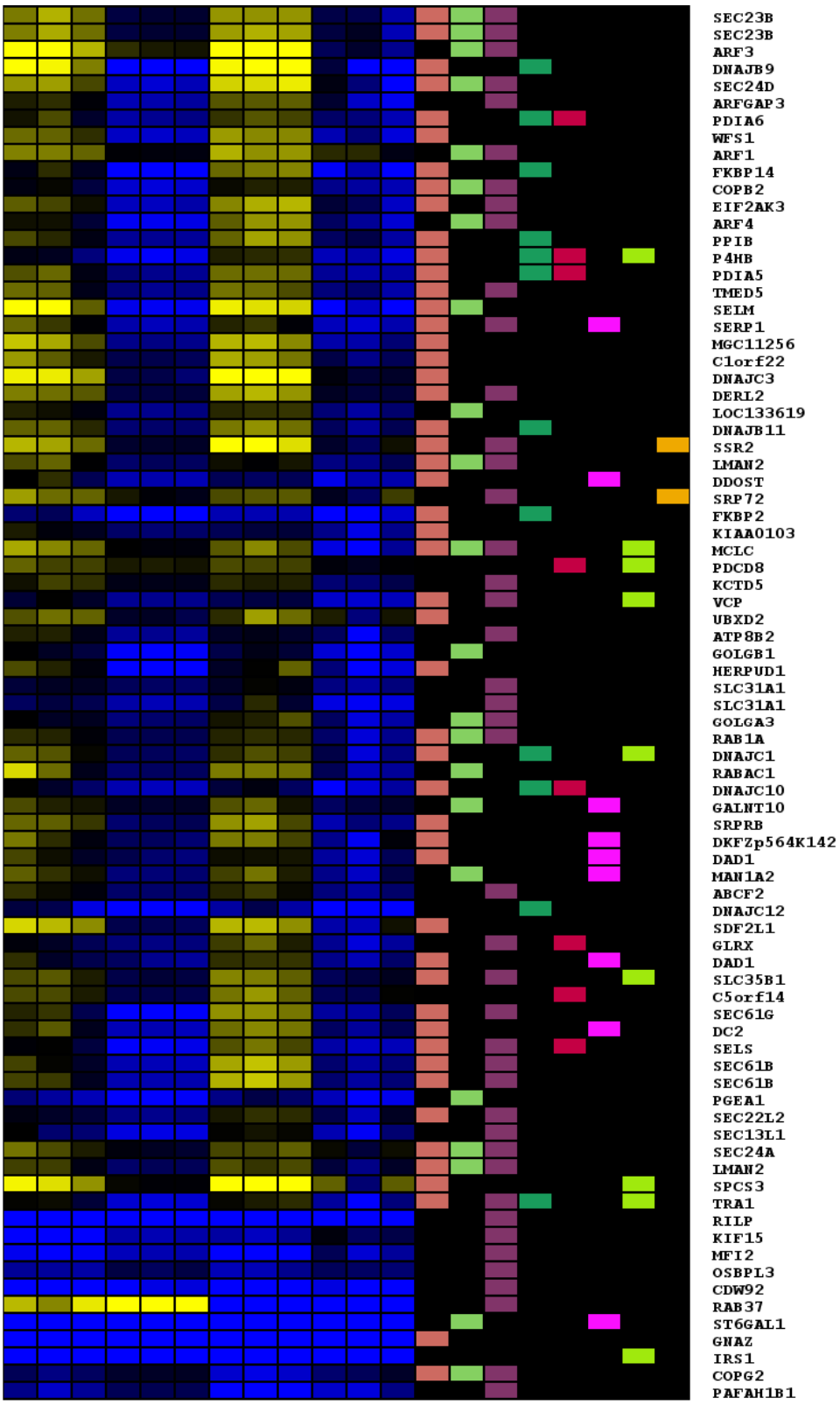

Figure 5-8 Gene expression changes in response to XBP-1s
Heatmap of significantly over-represented functional groups; consisting of upregulated or downregulated genes in both DEL and JSC-1 cells in response to XBP-1s overexpression. Each column represents one sample and each row one gene. Gene expression is shown as a pseudo-coloured representation of log(2) expression ratio with yellow being above and blue below the row/column median level of expression (set to 0) as shown by the scale. DAVID was used to annotate the genes into broad functional groups colour coded to right of heatmap.

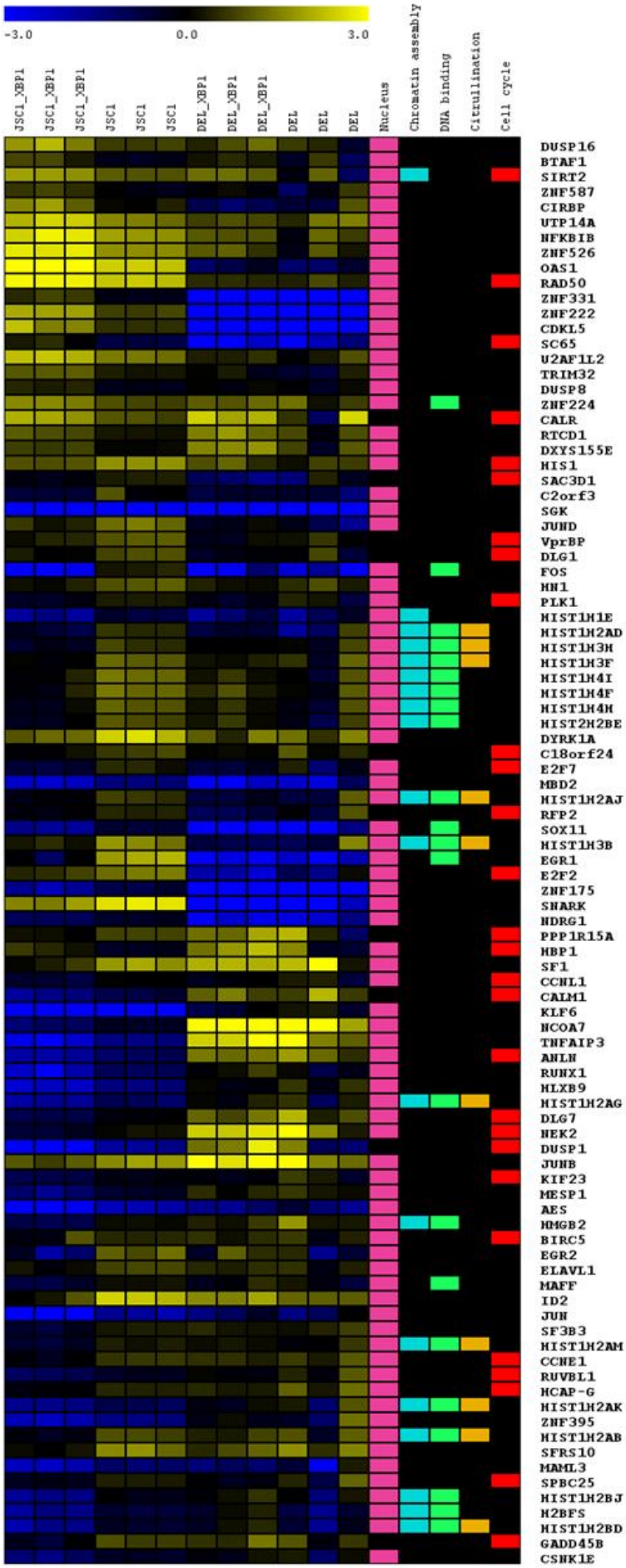

Figure 5-9 JSC-1 cell dependent gene expression changes in response to XBP-1s

Heatmap of significantly over-represented functional groups consisting of upregulated or downregulated genes found only in the JSC-1 cell response to XBP-1s overexpression. Each column represents one sample and each row one gene. Gene expression is shown as a pseudo-coloured representation of log(2) expression ratio with yellow being above and blue below the row/column median level of expression (set to 0) as shown by the scale. DAVID was used to annotate the genes into broad functional groups colour coded to right of heatmap.

Functional over-representation analysis of those genes significantly up- or downregulated in the comparison of XBP-1s overexpressing JSC-1 or DEL cells yielded 8 major significant annotation clusters including 229 genes; with 218 of those genes being upregulated and many belonging to more than one functional group (see Figure 5-8). Unsurprisingly, due to the known function of XBP-1s in the UPR; see section 1.3.3.1, the functions of these over-represented groups were related to the ER – 135 genes, Golgi – 79 genes, protein transport – 136 genes, protein folding – 27 genes, cell redox homeostasis – 16 genes, among others shown on the heatmap (Figure 5-8). The majority of these genes are involved in the secretory pathway and are important for preparing the cell for the production of large amounts of protein. These include genes encoding for proteins involved in targeting protein to the ER, the signal recognition proteins (SRPs) -19, -54 and -72. Proteins that are important for the next step of the secretory pathway, translocation of ER-targeted protein across the ER membrane into the lumen, are also upregulated including; signal sequence receptors (SSRs), SPR receptors, translocation associated membrane protein (TRAM1) as well as KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor-2 (KDELR2). Signal peptidase complexes (SPCs) are also found to be upregulated; these are required to cleave the signal peptide sequence from translocated proteins. A vast proportion of the genes shown to be upregulated in this comparison encode proteins required for protein folding in the ER including; several DnaJ/Hsp40-like proteins (DnaJb9, DnaJb11, DnaJc3), Hspa5/BiP, ERprotein44 (TXNDC4), FK506 binding protein-2 (FKBP2), peptidylprolyl isomerase B (PPIB), protein disulfide isomerase-associated-5 (PDIA5), ER oxidoreductins (ERO-1LB) as well as the hypoxia upregulated-1 (HYOU1) protein. Genes involved in the removal of misfolded protein from the ER by degradation pathways were also found including; ER degradation enhancer, mannosidase alpha-like-1 (EDEM), cAMP responsive element binding protein - 3 (CREB3) and homocysteine-inducible, endoplasmic reticulum stress-inducible ubiquitin-like domain member-1 (HERPUD1). Glycosylation is an important post-translation modification required by many

proteins for complete maturation and exit from the ER; for this reason several genes involved in glycosylation were upregulated in response to XBP-1 overexpression. These include members of the solute carrier family, dolichyl-diphosphooligosaccharide-protein glycosyltransferase (DDOST), mannosidase (MAN1A2) and ribophorin-I (RPN1). The remaining group of genes found to be induced by XBP-1 overexpression were those involved in ER-Golgi or endosomal trafficking which includes; members of the SEC24 related gene family, golgi transport-1 homolog B (GOLT1B), archain-1 (ARCN1), vesicle docking protein p115 (VDP), golgi SNAP receptor complex member-2 (GOSR2), coatomer protein complex, subunit epsilon (COPE), golgi reassembly stacking protein-2 (GORASP2), pallidin homolog (PLDN), golgi autoantigen (GOLGB1), lectin, mannose-binding-1 (LMAN1) and member RAS oncogene family (RAB1A).

Several genes including DnaJc3 ($P58^{IPK}$), DnaJb9 (ERdj4), DnaJb11 (HEDJ), EDEM, protein disulfide isomerases, and SERP-1 (RAMP4) were previously shown to be directly upregulated by XBP-1, and contribute to the general transcriptional changes seen when XBP-1 is supplied to fibroblasts or plasmablast like cells (Lee et al., 2003b) (Sriburi et al., 2007) (Wilson et al., 2007). This consistency with our data suggests that here we are observing typical XBP-1 response at the level of the whole transcriptome. Many of these genes were also identified during a similar study where gene expression changes of the cell line Raji were monitored in response to XBP-1s (Shaffer et al., 2004). This study also examined the gene expression profile of wild-type mature mouse B-cells in contrast with XBP-1 and BLIMP-1 deficient cells stimulated to differentiate by lipopolysaccharide (LPS) treatment. This confirmed that the changes seen due to ectopic expression of XBP-1s are comparable to the differentiation seen in response to a physiological trigger of B-cell terminal differentiation (Shaffer et al., 2004). Further study is required to determine if PEL and DEL cells overexpressing XBP-1s do become more ‘plasma cell like’. An increase in size and granularity of the cells was observed by FACs analysis, seen previously by Wilson *et.al.* (data not shown) (Wilson et al., 2007). However, further tests such as those carried out by Shaffer *et.al.* on Raji cells supplied with XBP-1s, including staining of the ER and measuring mitochondrial respiration, would aid further characterisation of cell phenotype

(Shaffer et al., 2004). Whether all the genes identified in this study are directly regulated by XBP-1s and the precise temporal regulation of gene expression will also require further study.

Although the genes identified by the DEL/JSC-1, XBP-1s overexpression comparison are dominated by those genes involved in the secretory pathway several genes that, so far, have not been implicated directly in the response to ER stress were also shown to be upregulated. These genes include glutaredoxin (GLRX), a thioltransferase dehydrogenase/reductase (SDR family) member-1 (DHOURS1) and the cyclin-dependent kinase inhibitor-1B (p27, Kip1). GLRX is an enzyme that catalyses deglutathionylation, a post-translational modification, which depends on the oxidative environment of the cell, and is important for sulfhydryl homeostasis and signal transduction (Gallogly and Mieyal, 2007). DHOURS1 is a member of a large protein family of NAD(P)(H)-dependent oxidoreductases and is therefore also involved in maintaining redox homeostasis (Wu et al., 2001b). Recently, a novel role for XBP-1 in the cellular response to oxidative stress has been reported, suggesting that these genes may be directly regulated by XBP-1 (Liu et al., 2009). XBP-1 dependent upregulation of the cell-cycle inhibitor p27 has not been reported; however, a reduction of p27 levels is associated with B-cell activation, which is then restored during the G1 cell-cycle arrest associated with B-cell terminal differentiation (reviewed in (Chen-Kiang, 2003)). PEL cells are also known to have a high level of p27 (see section 1.1.12.3). The identification of genes with no characterised role in the UPR and plasma cell differentiation indicates the potential for conserved functions of XBP-1s outside of the secretory pathway as suggested by a recent study by Acosta-Alvear and colleagues. In this study XBP-1s was shown to regulate, alongside its core group of ER function genes, genes involved in myogenesis as well as DNA damage and repair pathways; via a range of different sequence motifs (Acosta-Alvear et al., 2007).

A relatively small number of genes were downregulated in response to the expression of XBP-1s in both JSC-1 and DEL cells; this was to be expected as spliced XBP-1 is known to function as a transcriptional activator. Of the genes that were downregulated insulin receptor substrate (IRS1) was identified, this

may be linked to the repression of insulin signalling and increased insulin resistance associated with ER-stress (Ozcan et al., 2004).

Of those genes found to be significantly differentially expressed only in the JSC-1 comparison, 146 genes were upregulated and 220 were downregulated in a cell-type dependent manner. 94 of these genes, that may represent cellular genes modified by KSHV lytic gene expression in response to XBP-1 expression, were present in significant functionally enriched groups (see Figure 5-9). These groups were concerned functionally with the nucleus – 84 genes, chromatin assembly – 19 genes, DNA binding – 22 genes, citrullination (a common histone modification) – 10 genes and the cell cycle – 28 genes.

Due to the reported activity of the KSHV encoded SOX (shut off and endonuclease), a rapid and extensive shut off of host protein production is expected during KSHV lytic replication (Glaunsinger et al., 2005) (Glaunsinger and Ganem, 2004b). However, further microarray studies have since revealed that selective host transcripts can avoid SOX mediated degradation (Glaunsinger and Ganem, 2004a) (Chandriani and Ganem, 2007). In this study a dramatic downregulation of host transcripts is not seen and we believe this maybe due to the context of KSHV lytic replication. This appropriate induction of the KSHV lytic cycle in the B-cell environment does not result in the same extensive downregulation of host gene expression seen in the KSHV infection of TIME cells, although some similarities are seen (Chandriani and Ganem, 2007). This may be due to the activation of KSHV lytic replication, from latency, in the highly adapted and specialised environment of the plasma cell. In this physiologically relevant situation many of the transcripts are associated with ER stress and are refractory to translational arrest; potentially providing resistant to SOX degradation. In fact one of the genes shown to be upregulated in JSC-1 cells by XBP-1s-induced KSHV reactivation is the stress inducible cold inducible RNA binding protein (CIRBP). This protein is induced in response to a variety of cellular stresses, including hypoxia, and is important in promoting translation and stabilisation of mRNA; possibly providing a mechanism by which host transcripts can avoid SOX degradation (Lleonart, 2010). Further investigation into how KSHV moderates the host shut off to allow the accumulation of viral

transcripts is also required, and may indicate a potential mechanism for host transcript escape.

Interferon inducible transcripts 2',5'-oligoadenylate synthetase 1 (OAS1) and tripartite motif-containing protein 32 (TRIM 32) were also upregulated in the JSC-1 expressing XBP-1s analysis. Selective interferon induced genes were shown to escape SOX-mediated shut off in previous studies (Chandriani and Ganem, 2007). These genes in the absence of XBP-1s were already more highly expressed in JSC-1 cells compared to DEL; possibly because of the presence of KSHV in JSC-1 cells.

The dual specificity phosphatase-16 (DUSP-16) gene was also identified here as an upregulated gene during XBP-1s-induced KSHV reactivation in JSC-1. Previously, a related family member DUSP-5 was shown to be upregulated by KSHV lytic replication and evade SOX degradation (Chandriani and Ganem, 2007). This gene family is involved in the regulation of MAPK signalling, and members were identified earlier in this thesis as potential repressors of KSHV lytic replication by inhibiting JNK and p38 activity (see chapter 3 – reviewed in (Keyse, 2008)). The potential repressive action of DUSP-16 on reactivation is evident here due to an observed downregulation of the AP-1 pathway. Several of the AP-1 constituent proteins Jun, JunB, JunD, FOS and Maf are downregulated in response to XBP-1s overexpression in JSC-1 cells, as well as the stress inducible activator of the JNK pathway the growth arrest and DNA-damage-inducible- 45 beta (GADD45B) (Lopez-Bergami et al., 2010) (Liebermann and Hoffman, 2008) . During plasma cell development BLIMP-1 is known to upregulate the expression of pro-apoptotic GADD45, and plasma cells undergo rapid apoptosis if the correct survival signals are not provided (reviewed in (Shapiro-Shelef and Calame, 2005)). The control of the pro-apoptotic function of JUN is cell-type dependent, specific to the JUN binding partners available, and can be affected by both internal and external stimuli; including mitogenic and stress signals (reviewed in (Lopez-Bergami et al., 2010)). Therefore, the proposed downregulation of a potential apoptotic pathway during KSHV reactivation in differentiating B-cells suggested by the data presented here; indicates a potential mechanism for KSHV to maintain cell survival. The lytic protein vGPCR is associated with inducing cell proliferation

and oncogenesis is also known to activate the JNK pathway and induce transcription from AP-1 containing promoters (Bais et al., 1998) (Arvanitakis et al., 1997). Despite this, the highly controlled AP1 pathway terminal effector molecules are significantly downregulated in this analysis; possibly by the direct action of KSHV lytic replication in the environment of plasma cell differentiation, this therefore warrants further investigation.

Active JUN is also involved in the transcription of genes that regulate the cell cycle including cyclins D, A and E, but also cell cycle inhibitors. KSHV encodes a viral homologue of cyclin D during latency in order to drive cell proliferation (see section 1.1.12.3). During lytic replication the viral protein K-bZIP induces cell cycle arrest at the G1/S boundary, via upregulation of p21 and inhibition of both E2F proteins and cdk2/cyclinA/E complexes (Wu et al., 2003) (Izumiya et al., 2003b) (see section 1.1.13). However, this may also depend on the proliferation state of the host cell; for example, as B-cells terminally differentiate into plasma cells they enter G1-arrest and no longer divide (reviewed in (Chen-Kiang, 2003)). BLIMP-1 is known to downregulate the transcription factor E2F-1, which plays a major role in G1/S transition. Previous studies have shown that BCBL-1 cells synchronised in S-phase led to increased virus production when stimulated by TPA compared to non-synchronised cells (McAllister et al., 2005). This study suggests that the cell cycle is important in KSHV lytic replication and may be a particular target for viral lytic products. XBP-1s-induced reactivation of KSHV in JSC-1 cells led to a decrease in expression of two members of the E2F gene family (E2F2 and E2F7) as well as cyclin E (CCNE), suggesting a cell cycle block in G1 that is not seen in DEL cells. It would be interesting to investigate further the role of KSHV in manipulating the cell cycle during reactivation in PEL cells.

Related to this, the largest group of genes shown to be downregulated in JSC-1 cells expressing XBP-1s was the histone gene family. Histones are required during S-phase to package newly synthesised DNA into nucleosomes, the expression of histones are temporally restricted to the G1/S phase transition (reviewed in (Stein et al., 2006)). The alteration of chromatin structure has been identified as an important event in regulating KSHV gene expression and replication (reviewed in (Pantry and Medveczky, 2009)). However, this is

focused around epigenetic modification of histones for example histone acetylation which facilitates KSHV reactivation. Nucleosome positioning can also be altered and is required for KSHV genome replication. The depletion of histones as a mechanism of KSHV gene expression control has not been reported, however, our analysis indicates a possible KSHV or JSC-1 specific downregulation of histone encoding genes in response to XBP-1s overexpression.

5.3 Discussion

The aim of this investigation is to further understand the interactions between KSHV and the host cell during XBP-1s-induced reactivation, and secondly to explore the final stages of B-cell development. This, however, involved separating the two events that occur when XBP-1s is supplied to PEL cells, namely, plasma cell differentiation and KSHV lytic replication. The results obtained in this chapter have demonstrated that preventing RTA driven KSHV lytic replication is challenging and requires further development.

To dissect the role of KSHV lytic replication in PEL cells supplied with XBP-1s we attempted to block reactivation by targeting RTA. Previous studies had developed a JSC-1 cell line stably expressing shRNA-RTA that following TPA-treatment displayed an 80 % reduction in the population of cells expressing RTA. In this study we confirmed, by western blot analysis, that after TPA treatment detectable levels of RTA protein were not seen in JSC-1 cells expressing shRNA-RTA targeted to an invariant region of RTA sequence (JSC-1 50P-2 cells). By RT PCR analysis of the RTA transcript we showed that this shRNA acts as a miRNA, to block translation, and the JSC-1 50P-2 clonal cell line has maintained KSHV infection. RTA transcript in both JSC-1 and JSC-1 50P-2 cells is, as expected, was upregulated in response to TPA treatment, however, RTA protein is only detected in the absence of shRNA-RTA (see Figure 5-1).

This study requires RTA activity to be blocked in response to the physiological induction of KSHV reactivation by XBP-1s (Wilson et al., 2007). When XBP-1s was overexpressed in JSC-1 50P-2 cells a low level of RTA protein was detected by western blot. This is in contrast to the complete block to RTA production seen in JSC-1 50P-2 cells treated with TPA (see Figure 5-2 A). The ability of XBP-1s but not TPA to induce RTA expression in JSC-1 50P-2 cells is interesting, and the reasons for this are unclear. In chapter 3 we demonstrated that XBP-1s is able to directly bind to and transactivate the RTA promoter, whereas TPA induction occurs via the indirect action of PKC and the AP-1 pathway (see section 1.1.14) (Dalton-Griffin et al., 2009). XBP-1s induction of KSHV reactivation therefore appears to be more efficient. Despite low levels of

RTA protein seen in XBP-1s-expressing JSC-1 50P-2 cells, semi-quantitative RT PCR analysis of ORF29a/b indicates that the lytic replication seen in both JSC-1 and JSC-1 50P-2 is at similar levels (see Figure 5-2 B). Therefore, the shRNA-RTA-expressing PEL cell line system could not be used to investigate the action of XBP-1s in PEL in the absence of KSHV reactivation.

We were unable to determine whether shRNA-RTA could prevent reactivation in response to endogenous levels of XBP-1s due to the hypersensitivity of PEL to DTT treatment. PEL cells have a partially active 'physiological' UPR, however when this system is overwhelmed by the accumulation of large amounts of unfolded protein due to DTT or the proteasome inhibitor, bortezomib, cell death is induced (An et al., 2004). This analysis confirmed that XBP-1 is spliced in both PEL cell lines in response to DTT, however, this out of context activation cannot prevent cell death (see Figure 5-3) (Wilson et al., 2007).

Results obtained using the JSC-1 50P-2 cell line revealed that it was unsuitable for this study, as XBP-1s retains its ability to reactivate KSHV in the presence of shRNA-RTA. We therefore, attempted to develop and characterise a dominant negative form of RTA, described previously (Lukac et al., 1999). Although, expression of C-terminally truncated dominant negative RTA protein was able to reduce activity of full-length RTA in luciferase assay studies (see Figure 5-5), it could not prevent XBP-1s-mediated induction of the KSHV lytic cycle in either Clone 5 or JSC-1 cells (see Figure 5-6 B and Figure 5-7 B). These results indicate that the dominant negative RTA created is functional and able to inhibit RTA promoter transactivation by full-length RTA; however, this effect is not seen in KSHV infected systems. This is possibly due to the absence of RTA auto-regulation in the luciferase assay system. In this assay the amount of full-length and truncated RTA are equal and limited by the transfected input DNA. At this ratio, the dominant negative protein is able to either hetero-multimerise with the full-length RTA preventing its activity or homo-multimerise and effectively out-compete wild-type RTA for promoter binding (Lukac et al., 1999) (Bu et al., 2007). However, in Clone 5 or JSC-1 cells insufficient dominant negative RTA can be supplied to counteract the large amounts of RTA produced on induction, due to the positive feedback regulation of the ORF50 promoter. Unfortunately, the target sequence of the shRNA-RTA is present in

the dominant negative truncated form of RTA; therefore, analysis of the combined approaches on preventing KSHV reactivation could not be assessed.

We therefore pursued the comparison of the response to XBP-1s overexpression in the non-infected cell line DEL with that of KSHV infected PEL cells. This revealed a common gene list, with functions predominantly concerned with the secretory pathway (see Figure 5-8). This was expected due to the already well defined role for XBP-1 in preparing the cell for high-level immunoglobulin secretion during plasma cell development; see section 1.4.4 (Reimold et al., 2001) (Lee et al., 2003b) (Yoshida et al., 2003) (Shaffer et al., 2004). Interestingly, the upregulation of DnaJc3 ($P58^{IPK}$) is observed in both DEL and JSC-1 cells. This gene encodes an inhibitor of PERK and potentially explains how the UPR is selectively activated to increase the protein folding capacity, while avoiding translational arrest and ER-stress induced apoptosis during plasma cell differentiation (Yan et al., 2002) (see section 1.4.4).

This investigation provides an appropriate insight into the role of XBP-1 in plasma cell differentiation. A previous, similar study utilised the germinal centre derived, EBV infected cell line Raji, which does not provide the correct cellular transcriptional environment, namely $BCL\text{-}6^{-}$ and $BLIMP\text{-}1^{+}$, for the expression of XBP-1s. BLIMP-1 alongside XBP-1 is considered a master regulator of plasma cell differentiation and acts upstream of XBP-1 (Shaffer et al., 2002) (Shapiro-Shelef et al., 2003) (Shaffer et al., 2004). For this reason the BLIMP-1 dependent, XBP-1 independent genes, for example syndecan-1, were not reported by this study; as the expression of these genes is not significantly changed by XBP-1s expression in the already BLIMP-1 expressing DEL and PEL cell lines. Also, PEL cells have a partially active UPR, where ATF-6 is active and the XBP-1 branch is inactive (see section 1.4.4). PEL cells are trapped in a proliferating plasmablastic state prior to immunoglobulin secretion and therefore may not authentically represent the situation of a normal terminally differentiating B-cell. For example, ATF-6 has recently been shown to upregulated late during plasma cell differentiation and has many overlapping transcriptional targets with XBP-1s (Ma et al., 2010). Therefore many ATF-6 regulated genes may not appear in the normal temporal order in this analysis as they may already be upregulated in PEL (see section 1.3.3.1). To confirm that

the results seen are representative of normal plasma cell differentiation further study is required.

We could also investigate the role played by lytic KSHV during XBP-1s-induced B-cell differentiation. By determining the differentially expressed genes that were seen only in the JSC-1 analysis a list of potential novel candidates for KSHV host gene regulation was identified, providing prospective insights into KSHV biology (see section 5.2.5 and Figure 5-9). The functional annotations of these genes were related to processes concerned with cell proliferation and cell cycle. Previous gene expression analysis between KSHV negative lymphoma and PEL identified similar results, with many genes associated with the MAPK pathway being upregulated in KSHV positive PEL (Fan et al., 2005).

The host cell response seen to KSHV lytic replication will be highly dependent on the cell-type infected. For example the host response to KSHV reactivation in PEL may be very different to that in endothelial cells; as indicated by the lack of dramatic host shut-off seen in this study compared to that of KSHV infected TIME cells (Glaunsinger et al., 2005). Cell-type specific patterns of cellular genes were previously observed during a study of host gene induction in response to KSHV infection of endothelial, fibroblasts and B-cells (Naranatt et al., 2004). These differences will depend on the KSHV viral genes expressed as well as the biology and outcome of infection in these cell types. Differences in the host response would also be expected between studies examining changes seen on initial KSHV infection and those caused by reactivation. The transcriptional response to XBP-1s has also been shown to be cell-type dependent when secretory cells were compared to non-secretory cell types (Acosta-Alvear et al., 2007). However, many genes in this study and in previous studies overlap with our data suggesting a conserved host response to XBP-1s expression and KSHV reactivation.

6 General Discussion

The balance between latent and lytic replication is an important characteristic of herpesvirus biology and is necessary for persistent infection of the host. This is achieved by careful adaptation to the host, to facilitate exploitation of pre-existing cellular pathways in order to promote each stage of the virus life cycle. Latency is maintained by a variety of mechanisms employed by the virus to prevent detection by the host immune system (see section 1.1.12.). However, lytic replication is required to allow virus propagation within the host, and transmission to new hosts. Therefore, the switch between these phases must be tightly controlled to facilitate reactivation only in response to appropriate stimuli in the correct environment. In KSHV the expression of RTA, the 'lytic switch' protein, is highly regulated to provide the required level of life cycle phase control.

KSHV infects B-cells and we have demonstrated that reactivation is linked to plasma cell differentiation (Wilson et al., 2007). This is achieved by the direct activation of RTA by XBP-1s, a key transcription factor of the UPR (see chapter 3) (Dalton-Griffin et al., 2009). KSHV exploits the expansion of secretory apparatus in the absence of translational arrest, the physiological UPR that is associated with the terminal differentiation of B-cells to plasma cells. This situation provides an appropriate cellular environment for KSHV reactivation that assists efficient lytic replication and virion production at a suitable site for transmission, the lymphoid tissue of the oral mucosa.

Importantly, this mechanism of lytic reactivation appears to be conserved across herpesviruses, with a similar mechanism proposed, in this work, for other Rhadinoviruses, and for EBV by others (Laichalk and Thorley-Lawson, 2005) (Sun and Thorley-Lawson, 2007) (Bhende et al., 2007) (McDonald et al., 2010). Recently, reactivation from latency of the murine gamma-herpesvirus 68 (MHV68) was associated with plasma cell differentiation (Collins et al., 2009). Using the same system the MHV68 latency associated protein, M2, was shown to be capable of driving plasma cell differentiation. One proposed mechanism of M2-induced reactivation is the upregulation of BLIMP-1, the master regulator of plasma cell differentiation. Studies, again using the mouse model MHV68, have shown that BLIMP-1 is important for 'episodic' reactivation, as B-cells terminally

differentiate, and this is required to establish and 'renew latency reservoirs' for maintenance as well as a sustained humoral response (Siegel et al., 2010). These findings not only highlight the importance of this process in gamma-herpesvirus replication but also that it can be controlled by the virus itself (Liang et al., 2009). No M2 or 'reactivation conditioner' homologue has been identified in KSHV, however, a gene product with conserved function could explain why PEL cells are blocked at the plasmablast stage of B-cell differentiation. However, our data provides an indication that lytic replication of KSHV has an effect on the cellular gene expression profile during plasma cell differentiation, suggesting that KSHV manipulates this process further to achieve effective reactivation.

The UPR is a stress response that in the context of plasma cell differentiation acts in a physiological manner to allow the cell to function as a factory for antibody production. However, the UPR can also be activated in response to a variety of other cellular insults that lead to the accumulation of misfolded proteins, for example hypoxia (see section 1.3). It is therefore possible that many other cellular stresses may induce KSHV reactivation, by inducing XBP-1s or related 'stress' transcription factors', and this warrants further investigation.

Here we have shown that the RTA promoter contains response elements to the cellular stress inducible factors XBP-1s and HIF-1α, and responds directly to both. These transcription factors have been shown to have important and overlapping roles in both the response to cellular stress and normal development. It would be interesting to explore further the degree of overlap in their regulation of gene expression, as they share a response element core sequence 'ACGT'. This could lead to the possible identification of other convergent roles for these two stress-inducible factors.

The virus-host interactions outlined in this study are fundamental to KSHV biology and may be used to identify and explain potential therapeutic strategies for KSHV-associated disease.

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Appendix

Name	*Protein*	*Marker/Tag*	*Source*
pCDNA3 HIF-1α	HIF-1 α	None	Kind gift from Peter Ratcliffe (Raval et al., 2005)
pSIN-HABN-HIF-1 α/ pSIN-HA-HIF-1 α	HIF-1 α	NTD HA Tag	Backbone provided by Ed Tsao. HIF-1 α subcloned by candidate
p50LUC	4099bp of the ORF50 promoter including the first exon, intron and first seven codons of second exon	Luciferase reporter construct	Derived from DsRed reporter constructs from Sam Wilson (Wilson et al., 2007)
p50MUTLUC	Mutated version of above see Figure 3-8	Luciferase reporter construct	As above
pSIN-BNHA-DNRTA/ pSIN-DNRTA	C terminal truncated RTA (dominant negative)	CTD-HA Tag	Backbone provided by Ed Tsao. RTA truncated by PCR and subcloned by candidate
pDUAL-DNRTA-Cherry	C terminal truncated RTA (dominant negative)	mCherry	Backbone provided by David Escors. RTA truncated by PCR and subcloned by candidate
pHR-SIN-CSPW	shRNA (-HIF-1α/-RTA/-XBP-1/-GFP)	Puromycin acetylase	Backbone modified from CSGW by Sam Wilson. shRNA designed and cloned into EcoR1 site by candidate
pHR-SIN-CSHW	shRNA (-HIF-1α/-RTA/-XBP-1/-GFP)	Hygromycin phosphotransferase	Backbone modified from CSGW by Sam Wilson. shRNA designed and cloned into EcoR1 site by candidate
pXBPsIG	XBP-1s	eGFP/ His Tag	Generous gift of C. Tsantoulas generated under supervision of Sam Wilson (Wilson et al., 2007).
CTDHIS XBP-1s	XBP-1s	His Tag	Kind gift from Sam Wilson
pDUAL-XBP-1s - Cherry	XBP-1s	mCherry	Kind gift from Ed Tsao
pCMV RTA	RTA	None	Generously supplied by

			Adrian Whitehouse (Wilson et al., 2007)
pSIN-BNHA-kRTA	FL RTA	HA Tag	Kind gift from Ed Tsao
pSIN-BNHA-GFP/pSIN-GFP	GFP	HA Tag	Kind gift from Ed Tsao
pRpluc1-3087+s	3kb sequence upstream of RTA transcriptional start site and 1kb splice sequence of RTA	Luciferase reporter construct	Kind gift from Erle Robinson (Cai et al., 2006a)
pRpluc1115-1327	Contains the RTA promoter sequence containing HRE2 only	Luciferase reporter construct	Kind gift from Erle Robinson (Cai et al., 2006a)
pRpluc1-550	Contains the RTA promoter sequence containing HRE4/XRE only	Luciferase reporter construct	Kind gift from Erle Robinson (Cai et al., 2006a)